University of Alberta

Studies of the Mechanisms of Myelopoiesis
in Goldfish (*Carassius auratus* L.)

by

Barbara A. Katzenback

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences

Spring 2012
Edmonton, Alberta

Abstract

Development of progenitor cells into myeloid cells (i.e. macrophages and neutrophils) is critical to the survival of metazoans for maintenance of homeostasis and defense against pathogens. While much is known about myeloid cell development in mammals, less is known about this process in fish. The objective of my thesis was to characterize the myeloid progenitor cell subpopulations of goldfish, their growth factors, receptors and transcription factors (TFs) involved in the process of myeloid cell development (myelopoiesis).

Goldfish *kita* receptor and its ligand, *kitla*, were cloned, expressed and characterized. The *kita* was highly expressed in progenitor cells, compared to monocytes and mature macrophages. The recombinant KITLA was glycosylated, formed non-covalent homodimers, induced a progenitor cell chemotactic response and promoted the survival of progenitor cells. These results suggest a role for KITA/KITLA in the retention and survival of progenitor cells.

Colony stimulating factor-1 receptor (CSF-1R) is important for macrophage development. Antibody to goldfish CSF-1R was shown to specifically identify cells of the macrophage lineage, including a subpopulation of progenitor cells. The CSF-1R^{+} population of progenitor cells decreased with time of culture, coinciding with the generation of monocytes and macrophages.

The assessment of mRNA levels of a panel of TFs in progenitor cells showed that this cell population was committed to the macrophage lineage by day 2 of cultivation. Addition of rgKITLA and rgCSF-1 to progenitor cells modulated specific myeloid TF mRNA levels consistent with the functional characterization studies.

A procedure for the isolation of highly purified primary neutrophils was developed in this study. Primary neutrophils, isolated from the goldfish kidney produced, reactive oxygen intermediates and degranulated after exposure to mitogens and the fish pathogen, *Aeromonas salmonicida*, in a dose- and time-dependent manner. Goldfish granulocyte colony stimulating factor receptor (*gcsfr*) was identified and expressed by neutrophils. *gcsfr* mRNA levels were found to be significantly higher after exposure of neutrophils to mitogens or *A. salmonicida*, suggesting a role for GCSFR in neutrophil survival or activation during inflammation.

Taken together, my results highlight the importance of growth factors, receptors and transcription factors in the modulation of goldfish myelopoiesis.

Acknowledgements

I am happy to express my gratitude to a number of individuals whom have taken this journey with me. Please know that your guidance, support and encouragement throughout the duration of this thesis were greatly appreciated.

First and foremost, I would like to thank my supervisor, Dr. Miodrag (Mike) Belosevic. He has provided me with advice, helpful suggestions and many opportunities to grow as a scientist during the course of my studies. His dedication and enthusiasm towards science has been infectious, and his dedication, generosity, and kind mentorship towards his students have been inspirational - an example I will always try to follow. I fail to find the words to express how much your mentorship has meant to me. It has truly been a pleasure to work with you.

I would like to thank the members of my committee, Dr. Greg Goss and Dr. Catherine Field, and past members Dr. Larry Guilbert, and Dr. Mark Peppler, for their input and guidance during the course of this thesis. Your comments and suggestions were greatly appreciated. I would also like to thank my defense and candidacy examining members, Dr. Niels Bols, Dr. Daniel Barreda, and Dr. Brad Magor for interesting and thought-provoking questions and discussions. Dr. Allen Shostak has been an excellent source of constructive critism and statistical aid, both of which were highly valued.

Dr. Debbie Plouffe, Dr. James Stafford, and Dr. Patrick Hanington were exceptional mentors and I was lucky to have learned from each one of them. Thank you for taking the time to discuss science with me. To my past and present fellow lab mates Dr. John Walsh, Dr. Erick Garcia Garcia, Dr. Rebecca

Baldwin, Ayo Oladiran, George Haddad, Elaine Wilson, Jennifer Kerr, Steve Hitchen, Jonathan Pun, Jordan Hodgkinson, Mariel Hagen, Jiasong Xie, Dave Beauparlant, Scott Mitchell, Bharam Foroutanpay, Tamara Murray, and Matthew Karpman and as well as colleagues Dr. Kessen Patten, Dr. Chris Coutts, Dr. Martin Tresguerres, Ben Montgomery, Van Ortega, Herman Cortes, Kristina Petkau, Alysson Blaine, Hillary Vanderven, as well as many others, all of you have made the lab an exciting and fun place to work.

During the course of this thesis, I have had the opportunity to collaborate with a number of scientists. Dr. Greg Goss, Dr. James Stafford, Dr. Teruyuki Nakanishi, Dr. Daniel Barreda, Dr. Kathy Major, Dr. Domingo Miranzo Navarro, Dr. Ted Allison, Van Ortega and Aja Rieger have provided me with the opportunity to broaden my understanding of other research areas and have enhanced my growth as a scientist.

Lastly, my family and friends have been a key factor in the success of this thesis. My mother, Kathy, siblings Lorrie, Joey, Peter and Bonnie, brother in-law Justin and my partner Daniel, have been a constant source of support in all my endeveaours. My dearest friends, Stephanie, Shannon, Jacky, April, and Katie have always been there for retail therapy sessions, lunches, gelato, beers, runs, etc. I cannot tell you how much all of your support and kind words have helped me during the course of this thesis.

Table of contents

List of tables

List of figures

List of abbreviations

A-loop – activation loop
AA/aa – amino acid
ABC – ATP-binding cassette
AGM – aorta-gonad-mesenephros
ALM – anterior lateral mesoderm
ANOVA – analysis of variance
AO – acridine orange
AP – alkaline phosphatase
AST - astakines
ATCC – American Type Cell Culture
ATP – adenosine triphosphate
βc – beta chain common to IL-3 and GM-CSF receptors
BCA – bicinchroninic acid
BCIP – 5-bromo-4-chloro-3-indolyl phosphatase
BFU-E – blast forming unit-erythrocyte
bp – base pair
BrdU – bromolated deoxyuridine triphosphate nucleotides
BS^3 – bis (sulfosuccinimidyl) suberate
BSA – bovine serum albumin
bZIP – basic leucine zipper
CAF – citrate acetone formaldehyde
CaI – calcium ionophore
CCM – cell-conditioned medium
CD – cluster of differentiation
cDNA – complementary deoxyribonucleic acid
C/EBP – CCAAT-enhancer-binding proteins
CFU – colony-forming unit
CFU-GEMM – colony-forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte
CFU-GM – colony-forming unit-granulocyte/macrophage
CFU-IL-3 – colony-forming unit-interleukin-3
CFU-M – colony-forming unit-macrophage
CFU-Meg – colony-forming unit-megakaryocyte
CFU-S – colony-forming unit-spleen
CHO – Chinese hamster ovary cells
CHT – caudal hematopoietic tissue
CLP – common lymphoid precursor
CMP – common myeloid progenitor
ConA – convacalin A
COS-7 – monkey kidney fibroblast cell line
CRH – cytokine receptor homology
CRT - calreticulin
CSCs – cancerous stem cells
CSF-1 – colony-stimulating factor-1

CSF-1R – colony-stimulating factor-1 receptor
D – domain
DC – dendritic cell
DHR - dihydrorhodamine
DNA – deoxyribonucleic acid
dNTPs – deoxyribonucleic triphosphate
dpf – day post fertilization
E- embryonic day
EDTA – ethylenediaminetetraacetic acid
EGR/Egr – early growth response
EPO - erythropoietin
ERK-1/2 – extracellular signal-related kinases
FACS – flow cytometry activated cell sorting
FBS – fetal bovine serum
FCA – Freund's complete adjuvant
FIA – Freund's incomplete adjuvant
FITC – fluorescein isothiocyanate
GATA1 – GATA binding protein 1
GATA2 – GATA binding protein 2
GCSF – granulocyte colony-stimulating factor
GCSFR – granulocyte colony-stimulating factor receptor
GFP – green fluorescent protein
GM-CSF – granulocyte/macrophage colony stimulating factor
GM-CSFR – granulocyte/macrophage colony stimulating factor receptor
GMP – granulocyte/macrophage progenitor
GVHR – graft-versus host reaction
HA – hemagglutinin
HBSS – Hank's balanced salt solution
HEK – human embryonic kidney
HPC – hematopoietic progenitor cell
hpf – hours post fertilization
HRP – Horseradish peroxidase
HSC – hematopoietic stem cell
HSPC – hematopoietic stem/progenitor cell
ICM – intermediate cell mass
Ig – immunoglobulin domain
IL – interleukin
IL-3R – interleukin-3 receptor
IPTG- isopropyl b-D-1-thiogalactopyranoside
IRF – interferon response factor
JAK – Janus family of protein tyrosine kinases
Kbp – kilo basepair
kDa - kilo Dalton
KITLA – kit ligand a
KITLB – kit ligand b
Ks/Ka – synonymous/non-synonymous ratio
LMPP – lymphoid-myeloid primed multipotent progenitor
LPS – lipopolysaccaride
LT-HSC – long-term hematopoietic stem cell

MAPK – mitogen activated protein kinase
mCSF-1R – membrane bound colony-stimulating factor-1 receptor
MCSF – macrophage colony-stimulating factor
MEM – minimal essential medium
MEP – megakaryocyte/erythroid progenitor
MFI – median fluorescence intensity
MMP-9 – matrix metalloprotease-9
MPO - myeloperoxidase
MPPs – multipotent progenitors
MPX – myeloid-specific peroxidase
mRNA – messenger ribonucleic acid
mSCF – membrane bound stem cell factor
multi-CSF – multi-colony-stimulating factor
MW – molecular weight
Mya – million years ago
NBT – nitroblue tetrazolium
NCS – newborn calf serum
NETs – neutrophil extracellular traps
NF-IL-6 – nuclear factor interleukin-6
NF-κB – nuclear factor kappa B
NI – non-immune
NiNTA – nickel nitrilotriacetic acid
NK – natural killer cell
NMGFL-15 – incomplete medium used for goldfish primary kidney macrophages
NO – nitric oxide
nt - nucleotide
OD – optical density
PAS – periodic acid solution
PBS – phosphate buffered saline
PBLs – peripheral blood leukocytes
PCR – polymerase chain reaction
PDGFR – platelet derived growth factor receptor
PE – phycoerythrin
PGCs – primordial germ cells
PHA - phytohemaggultinin
PKC – protein kinase C
PKMs – primary kidney macrophages
PI – propidium iodide
PI3K – phosphatidylinositol-3-kinase
PLCγ – phospholipase C gamma
PLM – posterior lateral mesoderm
PMA – phorbal esters; phorbal-12-myristate-13-acetate
Poly I:C – polyinosinic-polycytidylic acid
P/S – penicillin/streptomycin
qPCR – quantitative polymerase chain reaction
RACE-PCR – rapid amplification of cDNA ends-polymerase chain reaction
RANKL – receptor activator of NF-kB ligand
RBCs – red blood cells

RBI – rostral blood island
RBLs – rat basophilic leukemia cell line
rgCSF-1 – recombinant goldfish colony-stimulating factor-1
rgKITLA – recombinant goldfish kit ligand a
rho - rhodamine
rgLIF – recombinant goldfish leukemia inhibitory factor
rgPECL-1 – recombinant goldfish prominin extracellular loop-1
RNA – ribonucleic acid
RNAi – ribonucleic acid interference
ROI – reactive oxygen intermediates
RT-PCR – reverse transcriptase polymerase chain reaction
SAS – saturated ammonium sulphate
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sca1 – surface cell antigen-1
SCF – stem cell factor
sCSF-1R – soluble colony-stimulating factor-1 receptor
SNAG – Snail/GFI1
SP – side population
SPF – specified pathogen free
sSCF – soluble stem cell factor
SOCS – suppressor of cytokine signaling
ST-HSCs – short-term hematopoietic stem cells
STATs – signal transducers and activators of transcription
TDL-15 – incomplete medium used in culture of trypanosomes
TDT – terminal deoxynucleotidyl transferase
TF – transcription factor
TICs – tumor initiating cells
TMAP – transferrin macrophage activating peptide
TMS – tricaine methane sulfonate
TSA – tryptic soy agar
TSB – tryptic soy broth
TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling
UTR – untranslated region
VEGFR – vascular endothelial growth factor receptor

Chapter 1: Thesis overview

1.1 Introduction

In 1882, Metchnikoff described starfish cells capable of engulfing, or phagocytosing, rose thorn shards. These phagocytic cells were termed macrophages, meaning “large-eaters”. Upon examination of vertebrates, Metchnikoff observed the same macrophage cells, but also described microphages, or “small-eaters”, now known as neutrophils. Metchnikoff’s documented the migration of these immune cells to the site of injury and their role in an inflammatory response. As a result, he proposed a cellular theory of immunity in which these phagocytes functioned as the central defense against foreign entities, reviewed by [1]. Together, Metchnikoff and Erhlich were awarded a Nobel prize in 1908 in recognition of their work on the cellular theory of immunity and the humoral theory of immunity, respectively.

The discovery of the hematopoietic stem cell (HSC) by the Canadian scientists McCulloch and Till in the early 1960s set forth basic principles of immune cell development: (1) an HSC can give rise to all cell lineages, and (2) the progeny are clonal [2-5]. Future studies focused on the production of macrophages and described the relationship between promonocytes, monocytes, and differentiated tissue macrophages. As a result of the developmental relationship between these cells, van Furth proposed the mononuclear phagocyte system theory to describe the system of macrophages and their precursors [6]. This theory was important in laying the foundation for the concept that cell types

undergo a progression of differentiation stages during their development, yet all belong to the same cell lineage.

Phagocytes are found in all metazoans and are required for the survival of the organisms. Macrophages and neutrophils are well known for their role in maintaining homeostasis through engulfment of dead or dying cells, wound repair, recognition and elimination of foreign pathogens, production of over one-hundred bioactive molecules, initiation of an inflammatory response, and resolution of the inflammatory response. However, myeloid cells need to be continually replaced. Therefore, the process of myeloid cell development, termed myelopoiesis, is essential to the survival of all metazoan organisms.

A number of model organisms have been used to study myelopoiesis, with the mouse model system being the focus of most research. Extensive research on myelopoiesis over the past 50 years has culminated in the identification of the site(s) of myelopoiesis, the progenitor cell types that give rise to mature myeloid cells, the extracellular and intracellular cues required, and a detailed understanding of the complex intracellular and extracellular milieu of factors that drive this tightly controlled process. When compared to the mechanisms of myelopoiesis in the mouse, studies using lower vertebrates (such as fish) and invertebrate models of development, have identified both evolutionary conservation as well as divergence in the mechanisms of myelopoiesis.

With over 30,000 identified species, teleosts are the most expansive class of vertebrates. Teleosts represent an excellent model system to study the evolution of vertebrate myelopoiesis as they are one of the ancient classes of vertebrates to retain the production of myeloid and lymphoid cell lineages that are

functionally equivalent to those of mammals. Using the goldfish as a model system, my thesis work focused on characterizing the progenitor cell population responsible for the production of myeloid cells and the extracellular and intracellular molecules that mediate teleost myelopoiesis.

1.2 Objectives of thesis

The main objective of my thesis research was to enhance our understanding of myeloid progenitor cell development in teleosts by (1) characterizing the goldfish myeloid progenitor cell population in terms of their growth factors, growth factor receptors, and intracellular transcription factors, and (2) functionally characterizing primary goldfish neutrophils and identifying the key growth factor receptor and transcription factors that influence their development.

The specific aims of my thesis were to:

1. Identify and characterize important growth factors and growth receptors involved in myelopoiesis and perform functional analysis of myelopoietic growth factors that influence progenitor cell development (Fig. 1.1).
2. Assess whether the different transcription factors can be used as molecular markers of goldfish progenitor cells, and examine how the mRNA levels of these transcription factors may be influenced by growth factors or pathogens (Fig. 1.1).
3. Isolate and characterize goldfish neutrophils through cytochemical staining and functional antimicrobial responses to mitogens and pathogens. Identify and perform molecular characterization of the primary

growth factor receptor and transcription factors important in neutrophil differentiation (Fig. 1.1).

1.3 Outline of thesis

This thesis is comprised of 9 chapters. Chapter 2 is a literature review focusing on myelopoiesis, the growth factors, receptors, and transcription factors involved, and how these systems compare between mammals and fish. Chapter 3 contains the detailed methodologies used to perform the research presented in this thesis. The identification and molecular characterization of goldfish *kita* and kit ligand a (*kitla*), along with the functional characterization of recombinant goldfish KITLA, can be found in chapter 4. The production of an anti-prominin antibody, validation of an antibody to goldfish colony-stimulating factor-1 receptor (CSF-1R) and the subsequent characterization of the progenitor cell population within the goldfish primary kidney macrophage cultures over time using the anti-CSF-1R antibody are described in chapter 5. The use of transcription factor expressions as markers of progenitor cells, and how the levels of transcription factor mRNA can be modulated in goldfish progenitor cells in response to time of culture, growth factors, or pathogens *in vivo* are presented in chapter 6. The isolation and functional characterization of neutrophils are described in chapter 7. The identification and molecular characterization of goldfish granulocyte colony stimulating factor receptor (GCSFR), and the transcription factor expression profile of neutrophils are presented in chapter 8. Lastly, a general discussion on the findings presented in this thesis, as well as ideas for future research on fish myelopoiesis are detailed in chapter 9.

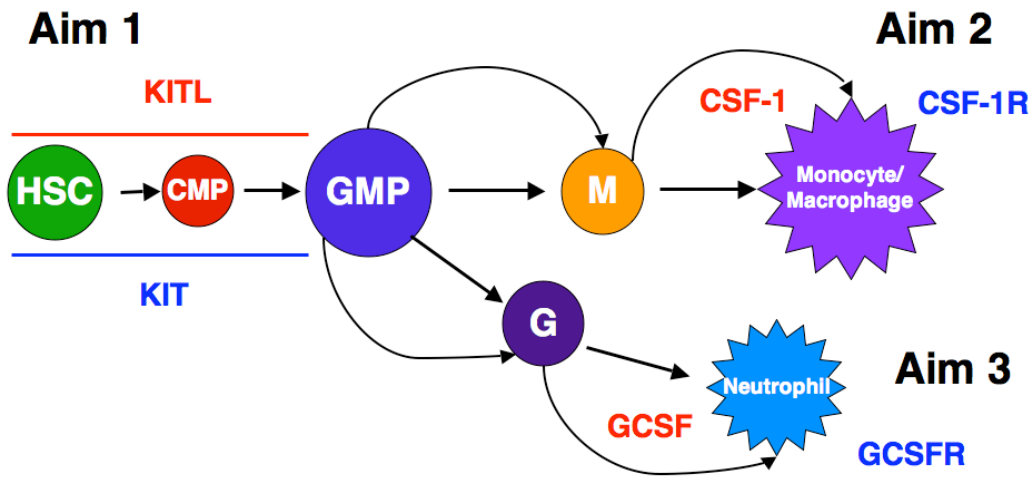


Figure 1: Schematic representation of thesis aims.
Growth factors are denoted in red lettering, growth factor receptors are shown in blue. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; M, committed monocytic progenitor; G, committed granulocytic progenitor; KITL, kit ligand; CSF-1, colony-stimulating factor-1; CSF-1R, colony-stimulating factor-1 receptor; GCSF, granulocyte colony-stimulating factor; GCSFR, granulocyte colony-stimulating factor receptor.

1.4 References

[1] **Cavaillon JM.** The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. J Leukoc Biol. 2011;90(3):413-24.

[2] **McCulloch EA, Till JE.** The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res. 1960;13115-25.

[3] **Till JE, Mc CE.** A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14213-22.

[4] **McCulloch EA, Till JE.** The sensitivity of cells from normal mouse bone marrow to gamma radiation *in vitro* and *in vivo*. Radiat Res. 1962;16822-32.

[5] **Becker AJ, Mc CE, Till JE.** Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963;197452-4.

[6] **van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL.** The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull World Health Organ. 1972;46(6):845-52.

Chapter 2: Literature review

2.1 Introduction

Hematopoiesis is an exquisitely fine-tuned, highly regulated, process whereby all blood cells develop from a small number of hematopoietic stem cells (HSCs). HSCs are characterized as long-term repopulating, pluripotent, quiescent cells that undergo symmetrical self-renewal to sustain the population of HSCs within the hematopoietic niche, or asymmetrical division to give rise to hematopoietic progenitor cells (HPCs) [1]. These HPCs can then develop along the lymphoid lineage, termed lymphopoiesis, to give rise to B-cells, T-cells, natural killer (NK) cells and dendritic cells (DCs). Alternatively, HPCs can develop along an erythroid lineage, termed erythropoiesis, to give rise to erythrocytes (red blood cells, RBCs) and megakaryocytes, or develop along a myeloid lineage to give rise to granulocytes (neutrophils, basophils, eosinophils, mast cells), mononuclear phagocytes (monocytes and macrophages), and DCs. The term myelopoiesis is collectively used to refer to the process of macrophage (monopoiesis) and granulocyte (granulopoiesis) development and will be used as such in this thesis. The cells from the lymphoid lineage represent the adaptive arm of the immune response, while cells from the erythroid and myeloid lineage represent the innate arm of the immune response. Regardless of lineage, the decisions made to commit and develop along a given lineage are controlled by extracellular growth factors and intracellular transcription factors that act in concert to regulate gene and protein expression to achieve the desired outcome. Recent studies have provided convincing evidence that hematopoiesis is

governed by an instructive, or growth factor driven model, rather than a permissive model system in which lineage outcome is dictated by stochastic means [2, 3], reviewed by [4].

Macrophages and neutrophils represent important phagocytic and antigen presenting cells of the immune system, and are of critical importance for survival of all metazoans in terms of maintenance of homeostasis and participation in host defense against invading pathogens. Thus, the continual production of myeloid cells within the hematopoietic niche is necessary during homeostasis and emergency conditions.

There are a number of critical junctures in myeloid cell differentiation, including the decision to commit to a common myeloid progenitor (granulocyte/macrophage colony-forming unit; CFU-GM) and the decision to commit to a macrophage or granulocyte lineage, thereby differentiating into a committed macrophage or granulocyte progenitor cell. The literature review that follows compares teleosts to the mammalian model systems and focuses on the site of myelopoiesis, and the growth factors, receptors and transcription factors that are involved in the commitment of an HSC to the myeloid lineage and subsequently to a committed macrophage or neutrophil progenitor cell, and the development of these cell types to their mature forms. Finally, a section on the goldfish model system used to study myelopoiesis is presented.

2.2 Site of hematopoiesis/myelopoiesis

2.2.1 Two waves of hematopoiesis in vertebrates

There are two waves of hematopoiesis in vertebrates. The first wave is primitive hematopoiesis and occurs during embryonic development. Definitive hematopoiesis follows primitive hematopoiesis and occurs in the post-natal or adult animal. Primitive and definitive hematopoiesis differ on a temporal scale, a spatial scale, and in the types of cellular progeny generated. With the exception of T-cells, which undergo final maturation stages in the thymus, lymphopoiesis and myelopoiesis occur in the major hematopoietic organs.

2.2.2 Primitive and definitive myelopoiesis in mammals

During embryogenesis, primitive hematopoiesis initiates in the yolk sac around E8.5 in the mouse [5]. These primitive hematopoietic cells transiently produce primitive erythrocytes and primitive macrophages, along with a number of megakaryocytes, reviewed extensively by [6, 7].

Discovered by McCulloch and Till, HSCs are capable of giving rise to clonal progeny of all types of hematopoietic lineages within the mouse bone marrow [8-11]. Based on the studies by McCulloch and Till, the irradiation of a host and transplantation of donor cells into a recipient to test for the long-term self-renewal of HSCs and their production of all blood cell lineages, or hematopoietic reconstitution, was set as the gold standard for identifying definitive HSCs. Definitive HSCs first arise in the embryonic mouse around E10 or E11 in the region known as the aorta-gonad-mesenephros (AGM), signaling the start of definitive hematopoiesis. The site of definitive hematopoiesis changes

to the fetal liver, fetal spleen, and then finally to the bone marrow where the HSCs reside for the life of the animal, reviewed by [5-7, 12].

2.2.3 Primitive myelopoiesis in teleosts

The development of myelopoiesis in fish has primarily been studied using the zebrafish model system. Primitive myelopoiesis is predominated by HPCs with primarily erythroid and myeloid development potential. Initially, primitive hematopoiesis is initiated in the anterior lateral mesoderm (ALM), that gives rise to the rostral blood island (RBI), and in the posterior lateral mesoderm (PLM), that gives rise to the intermediate cell mass (ICM). The RBI is the site of primitive myeloid cell development, generating primarily primitive macrophages that undergo rapid differentiation, lacking or having a very short monocytic stage [13] and a few neutrophils [14], while the ICM is the site of primitive erythroid cell development [15]. This stage of primitive hematopoiesis occurs early during zebrafish development, approximately 11 hours post fertilization (hpf). Following the onset of circulation, at around 24 hpf, the site of hematopoiesis then switches to the posterior blood island (PBI) [16] that produces multi-lineage progenitor cells capable of producing both primitive erythroid and myeloid cells [17]. Primitive macrophages act as phagocytes during tissue remodeling during embryonic development and in clearance of bacterial pathogens [13]. While primitive neutrophils also migrate to a site of infection, they were not observed to phagocytose bacteria [14]. The temporal, spatial and transcriptional control of zebrafish primitive hematopoiesis has been reviewed by [18-20]. Differences in the initial site of hematopoiesis occur between fish species, however, the

production of erythrocytes and macrophages during primitive hematopoiesis is consistent [21, 22].

2.2.4 Definitive myelopoiesis in teleosts

The onset of definitive myelopoiesis occurs around 36 hpf in the zebrafish. Here, HSCs seed the AGM and the caudal hematopoietic tissue (CHT) [23, 24]. By 48 hpf, the HSCs seed the kidney [23], the final hematopoietic site equivalent to mammalian bone marrow [25-27].

The existence of teleost kidney HSCs and HPCs capable of generating all hematopoietic lineages was demonstrated using transplantation studies in zebrafish and ginbuna crucian carp. Transplantation of whole kidney marrow from *gata1*^{eGFP} zebrafish into pre-thymic *vlad tepes* (*gata1*$^{-/-}$) zebrafish [27] or whole kidney marrow from β-*actin*^{eGFP} zebrafish into lethally irradiated zebrafish [28], resulted in rescue of the phenotype and produced lymphoid and myeloid cell types suggestive of the presence of HSCs capable of long-term reconstitution. However, these studies were complicated by the use of whole kidney marrow during transplantation.

The ginbuna crucian carp *(Carassius auratus langsdorfii*) has been developed as a unique model system for *in vivo* hematopoietic reconstitution experiments in teleosts. Female ginbuna crucian carp are triploid and represent a naturally occurring population of fish that undergo gynogenesis, essentially producing clonal offspring [29]. Triploid and tetraploid progeny can be produced when eggs are exposed to UV-irradiated or viable sperm from diploid male goldfish (*Carassius auratus* L.), respectively [29]. The production of triploid and

tetraploid offspring allows for the transplantation of triploid donor cells into tetraploid recipients without an immediate graft-versus host reaction (GVHR) [29]. Finally, donor and recipient cells can be distinguished from each other using DNA binding agents such as acridine orange (AO), propidium iodide (PI), and Hoechst 33342, to differentiate leukocytes with 3n and 4n DNA content [29-31]. The ginbuna carp model system is advantageous over the zebrafish model system, since relatively large numbers of HSCs and HPCs can be obtained from a single animal and transplanted into a recipient animal without an acute GVHR.

Using the ginbuna carp model system, the presence of teleost HSCs and HPCs were demonstrated, and HSCs were found to be associated with the renal tubules of the kidney [31-33]. The HSCs and HPCs were capable of engraftment and long-term production (>9 months) of all hemopoietic progeny, including erythrocytes, granulocytes, monocytes, thrombocytes and lymphocytes [31, 33, 34]. However, engraftment of donor HSCs and HPCs only occurred in anemia-induced or gamma irradiated recipients [30, 31, 34], and suggested that space within the hematopoietic niche was required for successful engraftment of HSCs to occur [31, 34].

Ginbuna carp HSCs and HPCs could be identified based on their ability to efflux dyes. HSCs, capable of long-term repopulation, could be isolated based on their efflux of Hoechst 33342, and were termed side-population (SP) cells [33, 35]. The ATP-binding cassette (ABC) transporter, ABCG2a, was found to be responsible for the SP phenotype in teleosts [32]. HSCs made up only 0.33% ± 0.15 of the total body kidney cells based on the SP phenotype, and were not found at appreciable levels in the head kidney, spleen, or blood [33]. A population of ginbuna carp HPCs could be isolated using rhodamine 123. Rhodamine (rho)

is effluxed by another ABC transporter, P-glycoprotein, and a population of HPCs capable of short-term reconstitution were found to be rho$^-$ [35].

Experiments using zebrafish and ginbuna carp provide strong evidence that the teleost kidney contains HSCs and HPCs capable of multi-lineage differentiation, including myelopoiesis (i.e. production of macrophages and neutrophils) [36].

2.3 Commitment to the myeloid lineage

2.3.1 Progression of cell development

The commitment of a pluripotent, self-renewing HSC to a common myeloid progenitor (CMP) is a progression of lineage fate decisions controlled by extracellular cues, such as growth factors, within the hematopoietic niche [3, 37, 38], as well as the modulation of intracellular transcription factors [39-42]. The process of committing to a CMP begins with long-term HSCs (LT-HSCs), capable of self-renewal and multi-lineage differentiation. LT-HSCs give rise to short-term HSCs (ST-HSCs) with limited capacity for self-renewal, which then differentiate into multipotent progenitors (MPPs) with no ability to self-renew, reviewed by [7]. The MPPs can give rise to the common myeloid progenitor (CMP) or the lymphoid-myeloid primed multipotent progenitors (LMPPs) [43-46]. The CMP can differentiate into megakaryocyte/erythroid progenitor (MEP) or to a granulocyte/macrophage progenitor (GMP) [47]. The LMPPs can differentiate into a common lymphoid precursor (CLP) that gives rise to T- and B-lymphocytes, or can also give rise to GMPs [43-46, 48, 49], and reviewed in [50].

HSCs and HPCs can be identified based on physiological properties, such as the efflux of dyes [33, 35, 51-53], or the expression of surface proteins, often involving the presence and absence of a multitude of different surface receptors to delineate hematopoietic cell lineage. In humans, primitive LT-HSCs are $CD133^+$, $CD34^-$, and give rise to HSCs that are $CD34^+$, which represent the majority of LT-HSCs in humans [54]. However, CD34 does not mark LT-HSCs in the mouse, and mouse HSCs are isolated based on the LSK expression - lineage negative (Mac-1, Gr1.1, Ter119, CD3, CD4, CD5, CD8, B220), surface cell antigen-1^+ ($Sca1^+$) and c-KIT^+ [55-59].

It should be noted that the paradigm of lineage commitment has recently been revised to the one described above. Under the old paradigm, the first decision an MPP made was to commit to either a common lymphoid or myeloid lineage. However, even the current hierarchy model of lineage fate decisions is a simplistic view of hematopoietic cell development. The factors that regulate cell fate decisions, such as the hematopoietic niche, cytokine signaling, and transcription factor modulation, are highly complex and we have yet to understand the process of hematopoiesis in its entirety. Refer to Fig. 2.2 for a schematic of the growth factors, receptors, and transcription factors involved in myelopoiesis.

2.3.2 Receptors and growth factors

The patterns of surface receptor expressions on stem/progenitor cells are utilized in mammalian systems to isolate subpopulations of cells. The expression of surface proteins on hematopoietic stem and progenitor cells (HSPCs) are for

interaction with the hematopoietic niche in terms of anchoring the cell, or for growth factor signaling. The role of the sialomucin CD34, PROMININ (CD133), c-KIT (CD117), interleukin-3 receptor (IL-3R), granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) and their cognate ligands will be discussed in this section.

2.3.2.1 CD34

CD34 is a type I integral membrane protein [60] belonging to the sialomucin family and is used as a marker of human HSPCs capable of hematopoietic reconstitution [61-64]. The gene and corresponding mRNA for CD34 has been identified in human [65], non-human primates [66], mouse [67], canines [68] and bovids [69, 70]. However, an orthologue of *CD34* has not been identified in teleosts, and will only be briefly discussed here.

In humans, the CD34 gene contains eight exons, is located on chromosome 1q32 and spans over 26 kbp [65, 71-73]. The mRNA transcript is 2615 bp which includes a 5' untranslated region (UTR) of 233 bp and a 3'UTR of 868 bp [60]. The open reading frame of the mRNA transcript encodes for a 373 amino acid protein with a signal peptide, extracellular domain, a transmembrane domain, and a cytoplasmic C-terminal tail [60]. The extracellular domain has 9 N-linked gylcosylations and numerous potential O-linked glycosylation sites [60]. The CD34 protein was predicted to be a 40 kDa protein, but is approximately 115 kDa as a result of heavy glycosylation [60]. The cytoplasmic tail has two sites for binding protein kinase C (PKC) and a potential site for tyrosine phosphorylation [60]. Additionally, an alternatively spliced variant of CD34 has been identified,

which splices in exon X between exons 7 and 8, encoding for a stop codon that results in a truncated cytoplasmic tail [74, 75]. The second variant of CD34 does not have motifs for PKC binding or tyrosine phosphorylation suggesting that it may signal differently [74, 75].

The similar genomic organization and protein structure of CD34 to podocalyxin, endoglycan [76] and the bird orthologue of podocalyxin, thrombomuccin [77], are members of what is now termed as the CD34 family, all of these proteins can be found on HSPCs, reviewed by [78]. A zebrafish podocalyxin-like protein has been predicted (accession number NM_001045016, NM_001025526), and may suggest the presence of other CD34 family members in teleosts. As members of the CD34 family are identified in teleosts, there will likely be a CD34 family member, perhaps the podocalyxin-like protein, which may be expressed on teleost HSCs, however, this remains to be determined.

The function of CD34 is unknown. However, CD34 is hypothesized to be a cell adhesion molecule for retaining HSPCs in the bone marrow niche, or it may play a role in preventing differentiation of HSPCs, reviewed by [78-81].

2.3.2.2 Prominin (CD133)

2.3.2.2.1 Prominin family members

Prominins have been identified throughout the metazoans, including human [82], rhesus monkey [83], mouse [82], rat [82], *Xenopus* sp. [84], teleosts [85, 86], *C. elegans* [87, 88] and *D. melanogaster* [82]. All prominins are pentaspan membrane proteins with an extracellular N-terminus, two small intracellular loops and two large extracellular loops that alternate, and an

intracellular C-terminus [82, 87-89]. The large extracellular loops are extensively glycosylated and have 6 to 11 potential N-linked glycosylation sites [83]. Members of the prominin family have a consensus sequence located in the second extracellular loop known as the prominin signature: CXPX(12,13)CX(5)(P/S)X(4)WX(2)hhXh, where X is any residue and number of residues, and h is any hydrophobic residue [82].

Three members of the prominin family exist in the metazoans, *prominin-1*, *prominin-2* and p*rominin-3*. *PROMININ-1* and *pROMININ-2* genes are found in mammalian vertebrates, whereas *prominin-1*, *prominin-2* and *prominin-3* genes are present in non-mammalian vertebrates [84]. Based on genomic analysis, it appears that *prominin-3* is only present in non-mammalian vertebrates [84], although it should be noted that *prominin-2* has yet to be identified in teleosts. Currently, it is proposed that an initial expansion of prominin genes in non-mammalian vertebrates occurred early during vertebrate evolution, and *prominin-3* was subsequently lost during mammalian evolution [84].

Each member of the prominin family has a number of alternative splice variants. Over 12 splice variants have been identified for *PROMININ-1* in humans and mice and a common naming system has been proposed to identify the different splice variants when comparing across species [90, 91]. Examination of the splice variants revealed changes in the extracellular N-terminus, the two large extracellular loops and in the intracellular C-terminus [91, 92]. Splice variants resulting in changes to the transmembrane regions or the intracellular loops were not observed. Although some prominin splice variants are common amongst mammals, other splice variants are species specific [91]. Splice variants are also specific to the tissue and the developmental stage [92-94].

2.3.2.2.2 Prominin distribution

PROMININ-1 protein was first identified by a monoclonal antibody, AC133, that bound to a specific glycosylated epitope expressed on $CD34^+$ cells, and decreased in surface expression as the cells underwent granulomonocytic differentiation [95]. Colony assays revealed that $CD34^+/AC133^+$, when compared to $CD34^+/AC133^-$ cells, contained a higher potential to give rise to granulocyte/macrophage colony-forming units (CFU-GM) [95]. Subsequent studies demonstrated that the AC133 could be used as a marker of stem cells from adult hematopoietic tissue [95, 96], embryonic stem cells [97], and tumors. It was present on tumor initiating cells (TICs), cancerous stem cells (CSCs), in prostate cancers [98, 99], brain tumors [100, 101], and other cancers as reviewed by [102-105].

In contrast to the restricted distribution of the AC133 antigen, *PROMININ-1* and *PROMININ-2* mRNAs are widespread. *PROMININ-1* mRNA was found in the pancreas, kidney, placenta, and at lower levels in the skeletal muscle, liver, lung, brain, bone marrow and fetal liver [89]. *PROMININ-2* mRNA was found to co-localize with *PROMININ-1* mRNA in tissues, however, *PROMININ-2* mRNA was absent from the retina [82]. PROMININ-1 and PROMININ-2 proteins were associated with cholesterol [106, 107] within membrane microdomains, regardless of cell type. Prominins are present in the plasma membrane protrusions in stem cells [96], the microvilli of epithelial cells [96, 108], the plasma membrane evaginations of rod photoreceptor cells [109] and oligodendrocytes [94].

2.3.2.2.3 Biological functions of Prominins

Currently, the ligand(s) and function(s) of the prominin family, their splice variants and differential glycosylation patterns are unknown. However, the interactions of prominins with other cell molecules and their potential capacity for intracellular signaling have been reported, with the research primarily focusing on PROMININ-1. Both PROMININ-1 and PROMININ-2 are known cholesterol-binding proteins in the plasma membrane [106, 107], and studies using mammalian PROMININ-1 have identified motifs in the extracellular and intracellular regions of the protein. Within the first extracellular loop, a predicted leucine zipper motif [83], and a ganglioside-binding domain capable of binding GM1 and GD3 were identified [110]. Furthermore, PROMININ-1 was shown to physically interact with protocadherin-21 and actin in the mouse retina [111].

Analysis of PROMININ-1 proteins predicted phosphorylation sites in their cytoplasmic tails [83]. In one study, the authors demonstrated that tyrosine 828 and 832 residues were phosphorylated in medullo-blastoma cell lines by Src and Fyn kinases [112]. Additionally, these phosphorylation sites were conserved in rat, mouse, chimpanzee and human [112], and suggest that phosphorylation of PROMININ-1 was important for its biological activity, and may have a role in signaling. However, the adaptor molecules or signaling pathways that occur as a result of PROMININ-1 phosphorylation are not known.

While the functions of prominins are unknown, their associations with protrusions from the plasma membrane suggest that they may be involved in maintainance and support of specialized cell structures. Studies have shown

frame-shift and missense mutations in PROMININ-1 proteins cause retinal and photoreceptor degeneration in mice and humans, and are associated with macular dystrophy in humans [109, 111]. The lack of cellular dysfunction in other organs was speculated to be the result of redundancy between PROMININ-1 and PROMININ-2.

Of particular interest, was the proposed role of PROMININ-1 maintaining an undifferentiated state in HSPCs [113, 114]. HSPCs were found to interact with osteoclasts through a micro-domain containing microvilli-like projections enriched with PROMININ-1 [113]. The HSPC microvilli-like projections seemingly penetrated the osteoclasts and appeared to transfer non-exosome punctate structures containing PROMININ-1 to the osteoclasts [113]. A recent study documented symmetrical distribution of PROMININ-1 in daughter cells during HSPC proliferation, and asymmetrical distribution of PROMININ-1 in daughter cells during HSPC differentiation suggesting that the loss of PROMININ-1 was required for differentiation of HSPCs to occur [114]. Within the HSPCs, PROMININ-1 was found within lipid rafts in protrusions of the plasma membrane, and in exosomal structures within the cytoplasm associated with the early endosome compartment [114]. HSPCs were found to release PROMININ-1 in exosomes as they differentiated, and the prominin-containing vesicles were taken up by mesenchymal stem cells [114]. Taken together, these data suggest a role for PROMININ-1 containing vesicles to act as intercellular communication bridges between HSPCs and their hematopoietic niche [113, 114].

2.3.2.2.4 Teleost prominin genes

Characterization of teleost prominins is limited to studies performed in goldfish and zebrafish, although prominin sequences have also been identified in *Takifugu rubripes* and *Tetraodon nigroviridis* [85]. A prominin-like transcript was first characterized in the goldfish [85]. Southern blotting predicted goldfish *prominin* to be a single copy gene with two alleles [85]. However, it is possible that these two proposed alleles could represent two *prominin* genes. Three prominin genes have been identified in zebrafish, termed *prominin-1a*, *prominin-1b*, and *prominin-3*. *Prominin-1a* and *prominin-1b*, located on chromosomes 14 and 1, respectively, are the orthologues of mouse *prominin-1*, and arose during the teleost genome duplication [86]. Zebrafish *prominin-3* is located on chromosome 13 and appears to be more closely related to human and mouse *PROMININ-2* than to human and mouse *PROMININ-1* [82, 86]. All of the teleost prominin proteins have the same characteristic structure of other prominin family members: a five transmembrane glycoprotein with two large extracellular loops and a prominin signature sequence.

2.3.2.2.5 Expressions of teleost prominins

The goldfish *prominin* transcript showed constitutive mRNA levels in the kidney, brain, gill, spleen, heart and intestine. However, slightly higher *prominin* mRNA levels were observed in the kidney, and prompted the analysis of *prominin* mRNA levels in the primary kidney macrophage subpopulations due to the hematopoietic nature of the teleost kidney. Analysis of goldfish *prominin* mRNA levels in sorted primary kidney macrophage subpopulations showed *prominin*

expression in all subpopulations, with highest expression observed in early progenitor cells and macrophages [85].

The *prominin-1a* is expressed as early as 1 hpf during zebrafish development and is highly expressed in the muscle, eye, brain and ovary tissues, with lower expression in the kidney, testis, gill and intestine [86]. The *prominin-1b* mRNA was detected mainly in the eye, and at low levels in the brain [86]. Both *prominin-1a* and *prominin-1b* mRNAs were associated with proliferating cell populations in the eye and brain [86] and were found within the photoreceptor cells in the retina of zebrafish [115]. A number of alternatively spliced variants of *prominin-1a* and *prominin-1b*, along with tissue specific glycosylation of prominin proteins were observed in the zebrafish, similar to what has been observed in mammalian systems [115]. Expression analysis of *prominin-3* in teleosts has not been reported. The biological role of prominins, their association with cholesterol in microdomains, and the ability to interact with other cellular proteins or their capacity to become phosphorylated are unknown in teleosts.

2.3.2.3 Stem cell factor and Kit receptor

2.3.2.3.1 Stem cell factor

Stem cell factor (SCF), also known as mast cell growth factor, steel factor and KIT ligand in mammalian systems, was identified by various groups [116-118] as short-chain four-helix bundle [119] encoded by the *Steel* locus in the mouse [120]. Mutants in the *Steel* locus were associated with defects in stromal cells, and resulted in reduced numbers of HSCs and HPCs in the context of hematopoiesis [121].

SCF is encoded on chromosome 12 in human [122, 123] and chromosome 10 in mouse [116, 117]. The *SCF* gene contains 9 exons, and transcription of the *SCF* gene produces two alternatively spliced mRNAs that differ in the presence or absence of exon 6 [117]. The mRNA encoding for the full length *SCF*, that includes exon 6, is a 2.2 kb transcript consisting of a 185 bp 5' UTR, a 819 bp open reading frame, and a 1.2 kbp 3' UTR [124]. Both *SCF* open reading frames encode for a 273 or 245 amino acid protein that has a signal peptide, an extracellular domain containing 4 irregularly-spaced cysteine residues and predicted to have 4 N-glycosylation sites, a transmembrane domain, and a cytoplasmic C-terminal domain [116, 124]. The SCF protein has been shown to be extensively glycosylated with both N- and O-linked sugars that make up approximately 30% of its molecular weight [125, 126]. The *SCF* splice variants, termed SCF^{248} (includes exon 6) and SCF^{220} (excludes exon 6), were expressed in the same tissues, however, the two isoforms have tissue specific regulation of expression [117]. The brain and bone marrow exhibit increased expression of SCF^{248} compared to SCF^{220}, while the spleen shows approximately equal levels of mRNA for both variants [117, 124].

While both of the SCF isoforms are produced as membrane bound forms (mSCF), they can also undergo proteolytic cleavage, likely by matrix metalloprotase-9 (MMP-9) [127, 128], to produce a soluble form of SCF (sSCF) [129, 130]. In human blood, sSCF is at a concentration of 3.0 ± 1.1 ng/mL [125]. Alternatively, mSCF may provide a means for cell-to-cell contact with the stromal cells in the hematopoietic niche [117], and may act to increase the signal strength provided to the HSC/HPCs, reviewed in [131]. Both mSCF and sSCF are capable of forming dimers [126, 132] and signal through their receptor, c-KIT.

2.3.2.3.2 c-KIT

The SCF receptor, c-KIT (CD117), was first identified as the cellular oncogene (*c-onc*) equivalent of the viral oncogene (*v-onc*), *v-Kit*, isolated from the Hardy-Zuckerman 4 feline sarcoma virus [133]. Studies mapped *c-KIT* to the *White* locus (*W*) in the mouse [120, 133], and demonstrated that mice with mutations in the *White* or *Steel* loci exhibit hypopigmentation, mast cell deficiency, macrocytic anemia, and sterility, while the complete loss of either of these genes was lethal [120, 134].

In humans, *c-KIT* is located on chromosome 4q11, and in the mouse *c-KIT* is located on chromosome 5q31 [135, 136]. The *c-KIT* gene is over 80 kbp and contains 21 exons [137]. Initiation of transcription produces a ~5.1 kbp *c-KIT* mRNA transcript that is widely expressed in mouse tissues, including embryonic brain, interstitial cells of Cajal, and renal tubules [138-142]. The *c-KIT* mRNA transcript consists of a 28 bp 5' UTR, an open reading frame of 2926 bp, followed by a 2144 bp 3' UTR [136], and similar organization has been observed in humans and cats [135, 136]. To date, *c-KIT* has been identified in a number of species including human [135], mouse [136], dog [143], cat [136, 144], cow [145], pig [146], chicken [147], *Xenopus* [148], and teleosts [149-151].

Based on structural analysis, the c-KIT protein was grouped within the Type III tyrosine kinase receptor family [135, 136] that includes colony stimulating factor-1 receptor (CSF-1R), platelet derived growth factor receptor (PDGFR), and FLT3/FLK2 receptor [136, 152, 153]. This family of receptors are characterized

by an extracellular domain consisting of five immunoglobulin-like domains and by an insertion of ~70 to 100 amino acids in the middle of the intracellular tyrosine kinase signaling domain [154]. The open reading frame of *c-KIT* encodes for a 976 amino acid protein with a signal sequence, an extracellular domain consisting of five immunoglobulin domains containing 12 cysteine residues and nine predicted sites for N-linked gylcosylations, a transmembrane region, and an intracellular carboxy-terminus tail containing a tyrosine kinase domain [135, 136, 144]. The c-KIT protein was predicted to be 110 kDa, but was found expressed as a 145 kDa protein, the result of N- and O-linked glycosylations [135, 144].

The c-KIT protein is primarily found on cells of the hematopoietic system and is a marker of HSCs capable of long-term reconstitution in humans [155] and mice [156-158]. Studies on the mouse hematopoietic system found c-KIT to be expressed on pluripotent and multipotent HSCs and myeloerythroid precursors, but not on differentiating or mature cell types [156-158], with the exception of mast cells [159]. Furthermore, long-term potentiating HSCs appear to exhibit an intermediate level of c-KIT expression, while HPCs have a high level of c-KIT on their surface [158]. Approximately 2×10^4 c-KIT receptors are found on normal human hematopoietic progenitor cells [160], and can undergo proteolytic cleavage, by MMP-9 or other proteases released by neutrophils [161], in the D5 domain of c-KIT [162], to release a soluble form of c-KIT [163]. Mast cells and endothelial cells, in addition to hematopoietic stem cells, can also produce a soluble c-KIT following proteolytic cleavage [163-165]. The concentration of soluble c-KIT receptor in human serum was determined to be 324 ± 105 ng/mL [166]. The soluble c-KIT receptor had a similar affinity to SCF as the membrane

bound c-KIT, and the soluble receptor is thought to regulate membrane bound c-KIT activity, *in vivo*, by blocking SCF binding [163, 167].

Four isoforms of c-KIT have been found in humans. Two variants differ based on the presence of the GNNK amino acid sequence in the juxtamembrane domain, and occur in human and mouse [168, 169]. Studies in immature myeloid progenitor cell lines transfected with GNNK- and GNNK+ c-KIT variants demonstrated functional differences. The hematopoietic cell lines transfected with the GNNK- c-KIT variant had enhanced growth and survival rates, was strongly and rapidly phosphorylated, and in turn, activated down-stream signaling pathways and initiated receptor internalization and degradation more readily compared to the hematopoietic cell line transfected with the GNNK+ c-KIT variant [170]. Early hematopoietic cell lines expressing the GNNK- isoform were capable of chemotaxis towards SCF, whereas early hematopoietic cell lines expressing the GNNK+ c-KIT isoform did not show a significant chemotaxis response [170]. The GNNK - c-KIT splice variant was the predominant form found expressed in mouse. The other two variants only occur in human, and differ in the presence of a serine residue in the interkinase domain [171].

Binding of SCF to c-KIT results in a number of down-stream signaling pathways. Two SCF monomers non-covalently associate in a “head-to head” manner to form a homodimer and binds to the second and third immunoglobulin domains of the c-KIT receptor with a high affinity [126, 172-176]. Binding of SCF to the D2 and D3 immunoglobulin domains of c-KIT results in receptor dimerization and conformational changes in the D4 and D5 domains of c-KIT. This results in autophosphorylation of the intracellular tyrosine kinase domains and downstream signaling [119, 177, 178]. A number of down-stream signaling

pathways mediate the action of SCF through c-KIT and include phosphatidylinositol-3-kinase (PI3K), phospholipase Cγ (PLCγ), members of the Janus family of protein tyrosine kinases (JAK) and signal transducers and activators of transcription (STATs), Src family members, the Ras/Raf/MAP kinase pathway, and others. The signaling pathway initiated depends on the cell type, and the strength and duration of the signal. Detailed reviews on the down-stream signaling pathways of c-KIT can be found in [179-181].

2.3.2.3.3 Biological functions of stem cell factor

SCF and its type III tyrosine kinase receptor c-KIT, are involved in hematopoiesis [131, 180, 181], spermatogenesis [182, 183], and development of melanocytes [183-185] and mast cells [164, 186-188].

Within the hematopoietic niche, one of the most important roles of SCF/c-KIT is to mediate HSC and HPC survival. An initial study showed c-KIT to be present on 7.8 + 0.6% of total mouse bone marrow cells [189]. Approximately 3.3 + 0.3% of the c-KIT^{+} cells were lineage negative, while the remaining c-Kit^{dull} cells co-expressed Gr-1 and Mac-1, markers for myeloid cells. Removal of the c-KIT^{+} cells and transplantation of the remaining cells to a lethally irradiated host showed a loss in spleen, IL-3, granulocyte/macrophage, and macrophage colony forming units (CFU-S, CFU-IL-3, CFU-GM, and CFU-M), suggesting that the c-KIT^{+} population of cells marks HSCs and myeloid progenitors [189]. Injection of mice with a blocking antibody to c-KIT caused a rapid decrease in numbers of the same CFUs upon transplantation, suggesting c-KIT was important for the survival of these cells *in vivo* [189]. Further studies have confirmed SCF/c-KIT mediate

the survival of long-term HSCs by blocking cell cycling or by inhibiting apoptosis [190, 191]. Furthermore, SCF is capable of synergizing with a number of different growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-1, IL-3, IL-6, and IL-7, among others, to promote the proliferation and differentiation of HPCs [192, 193] and reviewed in [174]. For example, SCF synergizes with IL-3 to promote the growth of CFU-GM [167], or can synergize with GM-CSF to promote cell proliferation [194]. Often, the progeny of HPC differentiation depends on the particular growth factor and SCF. Lastly, SCF acts as a homing signal to HPCs, such as CFU-GEMM (granulocyte-erythrocyte-macrophage-megakaryocyte), CFU-GM, CFU-Meg (megakaryocyte) and burst forming units-erythrocyte (BFU-E) [195]

The role of SCF and c-KIT is similar in spermatogenesis, melanocyte development and mast cell development in that SCF mediates the proliferation, differentiation, survival and migration of these cell types. During spermatogenesis, the Sertoli cells express membrane bound SCF and provide signals to the developing spermatogonia through c-KIT expressed on their surface. Interaction of mSCF with c-KIT promotes the differentiation and survival, through the inhibition of apoptosis, of the developing spermatogonia. The action of SCF and c-KIT during the development of spermatogonia, and the signaling pathways involved are reviewed in [182, 196]. Melanocytes express c-KIT on their surface and receptor activation is necessary for the proliferation, survival and migration of the melanocytes to the periphery from the neural crest, and is reviewed in [197]. Within the hematopoietic niche, SCF signaling suppresses the production of MMP-9 in mast cell progenitors, and is thought to retain the

progenitors in the niche [198]. The action of membrane bound and soluble SCF mediates the proliferation, differentiation, migration and survival of mast cells and has recently been reviewed in [159]. Furthermore, SCF activates mast cells in a number of tissues. Reviews on mast cell activation by SCF can be found in [199, 200]

2.3.2.3.4 Teleost Kit and Kit ligand

Whole genome duplication has resulted in two orthologues of *c-KIT* and *SCF* in teleosts. Teleost orthologues of *c-KIT,* termed kit a (*kita*) and kit b (*kitb*), were first identified in zebrafish and have subsequently been predicted from genomic analysis of *Takifugu rubripes*, and *Tetraodon nigroviridis* [149, 151]. The *kita* orthologue has also been identified and characterized in *Carassius auratus* L. [150] (see chapter 5). The two orthologues of mammalian *SCF* are termed kit ligand a (*kitla*) and kit ligand b (*kitlb*) [149, 201]. The two orthologues, *kitla* and *kitlb*, have been identified in zebrafish, and predicted in fugu, medaka, and stickleback genomes [201]. The *kitla* orthologue has also been identified and characterized in goldfish [150] (see chapter 5).

Zebrafish *kita*, located on chromosome 20, and *kitb,* located on chromosome 1, are the orthologues of human and mouse *c-KIT* [149, 151]. Both *kita* and *kitb* genes contain 21 exons, however, their respective proteins only retain 55% identity to each other [151]. The partitioning of gene distribution and function was proposed to explain the retention of *kita* and the duplicated gene, *kitb* [149, 151]. From studies on developing zebrafish, *kita* is expressed in hematopoietic progenitors, melanoblasts and melanocytes derived from the neural crest, along the lateral line, the notochord and pineal gland [149, 151]. The

expression of *kitb* occurs by 9 hpf and does not overlap that of *kita*. Instead, *kitb* expression is restricted to the Rohon-Beard neurons, trigeminal ganglia, and otic vesicle [151]. Together, the expression of *kita* and *kitb* approximates that of *c-KIT* in the mouse model system, with the notable exception of *c-KIT* expression in primordial germ cells (PGCs).

The *kitla* gene is located on chromosome 25 and the *kitlb* gene is located on chromosome 4 of the zebrafish genome [202]. *Kitla* has 9 exons while *kitlb* has 8 exons [201]. The nine *kitla* exons correspond to the 9 exons of mammalian SCF isoform 1, including exon 6 which allows for cleavage of membrane bound SCF into a soluble form [201]. However, *kitlb* appears to correspond to SCF isoform 2, in which exon 6 has been spliced out. The expression of *kitla* is first observed at 19 hpf in the zebrafish and is found in the developing tail bud, pineal gland, sensory epithelium of the ear, ventral otic vesicles, and in the somites [201]. Similar to the expression of goldfish *kita*, *kitla* showed constitutive mRNA levels in tissues [150] and this expression pattern was similar to what was observed in adult zebrafish tissues [202]. Goldfish *kitla* showed high levels of mRNA in isolated putative progenitor cells and monocytes compared to macrophages [150]. Zebrafish *kitlb* mRNA expression was observed in the brain ventricles, ear and cardinal vein plexus and at lower levels in the skin as zebrafish development progressed [201].

2.3.2.3.5 Biological functions of teleost kit receptors

Based on the non-overlapping expression of *kita* and *kitb*, the functional roles of c-KIT in mammals may be partitioned between teleost KITA and KITB. The zebrafish mutant *sparse,* shown to map to *kita* [149], or kit^{w34} mutants [203]

show defects in their pigmentation pattern. Zebrafish KITA was shown to be involved in the dispersion and maintenance of melanocytes [149], and may play a transient role in melanocyte differentiation when melanoblast development is perturbed [204]. Furthermore, knock-down of zebrafish *kitla* or *kitlb* using morpholinos supported the involvement of KITLA in the migration and survival of melanocytes [201]. Teleost *kit* expression in melanocytes has been implicated in the pigment pattern formation in a number of fish species [149, 205-207] and suggests that the functions in myelocyte development have been partitioned to the *kita* orthologue.

The role of teleost *kita/kitla and kitb/kitlb* during hematopoiesis is not clear. Examination of hematopoiesis in zebrafish *sparse* mutants revealed no obvious defects in hematopoiesis during development. Although, slight decreases in promyelocyte and neutrophil cell numbers, and slight increases in band cells and monocytes were observed in the kidney [149]. In addition, zebrafish injected with *kitla* morpholinos or *kitlb* morpholinos also did not show defects in hematopoiesis. However, studies in the goldfish model system (described in chapter 5) demonstrated the expression of *kita* mRNA in isolated kidney progenitor cells, and the functional role of goldfish KITLA in progenitor cell chemotaxis, proliferation, and maintenance [150]. Taken together, these data suggest that KITA and KITLA proteins play a central role in myelopoiesis. However, redundancy between the two ligands and receptors may account for the absence of hematopoietic defects in the zebrafish system, or there may be redundancy with another tyrosine kinase receptor. Additionally, the absence of hematopoietic defects in the zebrafish may represent KIT-independent and KIT-

dependent stages of hematopoiesis. The function of KITLB and KITB during hematopoiesis in teleosts remains to be determined.

Lastly, c-KIT plays a role in the development of primordial germ cells (PGCs) in mice. Examination of primordial germ cell development in fish revealed that *kita* and *kitb* expression was not detected in PGCs, and suggests teleost KITs do not play a role in the development of PGCs [149, 151]. However, it appears that *kita*, *kitb*, *kitla* and *kitlb* play a role in ovarian folliculogenesis in zebrafish and provides evidence of neofunctionalization of these genes [202].

2.3.2.4 Interleukin-3 and Interleukin-3 receptor

2.3.2.4.1 Interleukin-3

Interleukin-3 (IL-3) is a multi-lineage colony-stimulating factor (multi-CSF) that acts on multipotent HPCs, with erythroid and myeloid potential, to promote their proliferation and differentiation [208-210]. The gene encoding IL-3 has been identified in human and non-human primates [209, 211], mice [212], rats [213], bovids [214] and ovids [215]. However, genes encoding IL-3 and the specific IL-3 receptor alpha (IL-3Rα) have not been identified in any teleosts to date, despite genome sequencing. The lack of IL-3 in teleosts may be due to difficulties in identifying the IL-3 orthologue in teleosts due to the low sequence conservation of IL-3 observed between mammals, or may represent the evolutionary loss of IL-3 in teleosts. As IL-3 and IL-3R have not been identified in teleosts, IL-3 and IL-3R will only be briefly discussed here. The structure, function and regulation of IL-3 and its receptor have been extensively reviewed by [216-218].

Found on human chromosome 5q23-31 [219] and mouse chromosome 11 [220], the *IL-3* gene contains 5 exons and four introns [209]. The human *IL-3* mRNA transcript is approximately 1 kbp and has an open reading frame of 456 nt that produces a 152 aa precursor protein [209]. The IL-3 precursor protein contains a 19 aa secretion signal peptide that is cleaved to produce a 14.6 kDa protein with 2 N-linked glycosylation sites [209]. Variable glycosylation of human IL-3 leads to molecular weights of the protein ranging from 14.6 to 30 kDa [209]. The mouse *IL-3* gene is structurally similar to the human *IL-3* gene and encodes for a 166 aa protein containing a secretion signal peptide to produce a 22 kDa protein with four predicted N-linked glycosylation sites [212, 221]. Despite the conserved features of the human and mouse IL-3, there is only 29% aa homology between human and murine IL-3 [209], and may suggest evolutionary pressure on this early-acting hematopoietic cytokine [211]. IL-3 is a member of the short-chain 4-α-helical bundle subset of cytokines, and other members of this family include IL-2, IL-4 IL-5, GM-CSF and macrophage colony stimulating factor (M-CSF = CSF-1) [217, 222, 223]. The 3D structural analysis of murine IL-3 demonstrated heterogeneity in the IL-3Rα binding site, which may facilitate binding to the two IL-3Rα isoforms [223], discussed below. Interestingly, *IL-3*, *IL-4*, *IL-5* and *GM-CSF* are all found on chromosome 5q in humans. The close proximity of the CSFs on the chromosome, along with their similar structure and function may suggest they arose from a common ancestral gene [224].

2.3.2.4.2 Interleukin-3 receptor

The interleukin-3 receptor is a heterodimer comprising of the IL-3 receptor alpha (IL-3Rα) and a common beta-receptor (βc) [225]. The human *IL-3Rα* is located on chromosome Xp22.3 and Yp11.3 and is found close to the *GM-CSFR* gene [226, 227], while in mouse, the *IL-3Rα* gene is located on chromosome 14 [228], and is separated from the mouse *GM-CSFR* gene. The human IL-3Rα is produced as a 378 aa precursor protein that contains an 18 aa signal peptide, a 287 aa extracellular domain that contains four conserved cysteine residues and the characteristic WSXWS motif (LSXWS) of the hematopoietin receptor family, a 20 aa transmembrane domain followed by a 53 aa intracellular domain [225]. Although the IL-3Rα chain is predicted to be 41 kDa, it is found to have a molecular weight of 70 kDa due to the 6 potential N-linked glycosylations that are present on the extracellular domain [225]. Recently, a new isoform of IL-3Rα (SP2) has been discovered which is an alternative splice variant lacking domain 1 of the extracellular domain of the receptor through the exclusion of exons 3 and 4 [229]. SP2 is predicted to be 31 kDa, and has 4 potential N-linked glycosylation sites that give SP2 an apparent molecular weight of 41 kDa [229]. Both SP1 and SP2 are expressed in the same cell, however, the mRNA levels of SP2 are lower than that of SP1 and are not always translated to protein [229]. Differential growth and differentiation responses were observed when IL-3 associated with SP2 compared to that of SP1, but was dependent on the cell line examined [229], and may be mediated through binding of different epitopes of the IL-3 ligand depending on the isoform of IL-3Rα [230].

In humans, the gene for the βc subunit is on chromosome 22q12.2-13.1 [231]. However, mice have two genes, a βc subunit and an IL-3 specific βc subunit (βc_{IL-3}), both located on chromosome 15 [232]. The 120 kDa βc receptor has an extracellular domain containing two hematopoietin domains, each with four conserved cysteine residues and the WSXWS motif, with three potential N-linked glycosylation sites, a transmembrane domain, and an intracellular domain that has two signaling motifs termed box 1 and box 2 [218, 233]. Crystallization of the βc receptor revealed that the βc are homodimers, and associate with the receptor alpha chain [234] to initiate signaling. Recently, it has been proposed that IL-3/IL-3Rα forms dodecameric complexes. This occurs when IL-3 binds the IL-3Rα, two of the IL-3/IL-3Rα complexes bind to the homodimeric βc, forming a hexamer. Two of these hexamers then associate to form a dodecameric complex [235].

The low affinity IL-3Rα binds IL-3, and this complex then associates with the βc chain to form a complex with high affinity for IL-3. The action of IL-3 binding to the IL-3Rαβc results in activation of a number of signaling pathways including JAK/STAT, mitogen-activated protein kinase (MAPK) and PI3K, reviewed by [217].

2.3.2.4.3 Biological functions of IL-3/IL-3Rα

IL-3, produced by activated T-cells [236], acts on multipotent erythro/myeloid HPCs to promote their self-renewal and proliferation and can act on committed myeloid progenitors to promote their proliferation and differentiation [208-210, 237, 238]. Injection of mice with recombinant IL-3 induced an increase

in numbers of blood monocytes, neutrophils and eosinophils, as well as an increase in the number of progenitor cells and developing myeloid cells in the spleen [239]. A similar effect was observed when IL-3 was administered *in vivo* to non-human primates [240].

Studies using knock out mice have provided insight into the function and redundancy of IL-3 and its receptor. βc deficient mouse appear to be normal with the exception of impaired eosinophil development and pulmonary lung disease, and were unable to respond to challenge with the parasite *Nippostronylus brasiliensis* [241]. HPCs from βc deficient mice did not respond to treatment with GM-CSF or IL-5, but retained the ability to respond to IL-3, likely due to the action of IL-3 through the mouse specific $\beta c_{IL\text{-}3}$ receptor [241]. However, $\beta c_{IL\text{-}3}$ deficient mice were normal, and HPCs were capable of responding to IL-3, GM-CSF, and IL-5, suggesting that signaling through βc and $\beta c_{IL\text{-}3}$ is redundant [241]. To mitigate the functional redundancy observed between IL-3, GM-CSF and IL-5, double mutant mice deficient in the βc receptor and IL-3 ligand were generated. As with the βc deficient mice described previously, similar pathology was observed including reduced numbers of eosinophils [242]. However, double mutant mice displayed normal hematopoiesis and recovery following challenge with the pathogen *Listeria monocytogenes* [242]. Taken together, these studies suggest the existence of either a different ligand-receptor pair, or an alternate signaling mechanism that mediates the survival, proliferation and differentiation of early multipotent myeloid HPCs and their committed progenitors.

2.3.2.5 Granulocyte-macrophage colony-stimulating factor/Granulocyte-macrophage colony-stimulating factor receptor

2.3.2.5.1 Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) shares redundancy with IL-3 in terms of its function. However, GM-CSF acts on a more mature population of HPCs and has been associated with the formation of both granulocyte and macrophage colonies from CFU-GM [243, 244]. The gene for *GM-CSF* has been identified in human [244], murine [245, 246], bovine [247], ovine [248], porcine [249], and woodchuck [250] species. However, similar to that of *IL-3*, *GM-CSF* has not been identified in teleosts. The close proximity of *IL-3* and *GM-CSF* on the same chromosome may suggest that a genomic deletion occurred on this chromosome, subsequent to the divergence of fish and mammals.

The *GM-CSF* gene spans 2.5 kbp and consists of 4 exons [251, 252], located on chromosome 5 in humans [224, 251, 252] and chromosome 11 in mice [245, 251, 253]. Transcription of mouse *GM-CSF* produces a ~1.2 kbp transcript that encodes for a 153 aa precursor protein. Following cleavage of the signal peptide, the 124 aa protein is predicted to be 14.1 kDa [246], however, the apparent molecular weight of mouse GM-CSF ranges from 22-33 kDa, presumably due to protein glycosylation [243]. Human GM-CSF is a 144 aa protein with the mature protein predicted to have a molecular weight of 14.5 kDa [244]. As mentioned previously, GM-CSF also falls within the short-chain 4-α-helical bundle family of cytokines [217, 222, 223], see IL-3 section.

2.3.2.5.2 Granulocyte-macrophage colony-stimulating factor receptor

The granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) is composed of heterodimeric alpha and beta chains as described for IL-3. The *GM-CSFR*α chain is found on chromosomes Xp22.32 and Yp11.3 in humans [254, 255] and chromosome 19 in mice [256]. The 400 aa precursor GM-CSFRα protein is comprised of a 22 aa signal peptide, 297 aa extracellular domain with 11 potential N-linked glycosylation sites, a 27 aa transmembrane domain, and a 54 aa cytoplasmic domain [257]. GM-CSFRα is structurally similar to IL-3Rα, and contains an immunoglobulin domain (Ig), the four conserved cysteine residues and the WSXWS motif characteristic of the hematopoietin receptor family [257]. Binding of GM-CSF to the Ig domain of the GM-CSFRα facilitates the formation of the low affinity GM-CSF/GM-CSFRα complex [258] that can then associate with the βc receptor, previously described in the IL-3 receptor section. The GM-CSF/GM-CSFR complex also forms a dodecameric complex [259] that leads to signal transduction [260]. Since the βc chain is common to IL-3, IL-5 and GM-CSF, the βc chain signals through JAK/STAT, MAPK, and PI3K pathways [217, 260].

2.3.2.5.3 Biological functions of GM-CSF/GM-CSFR

GM-CSF is produced by activated T-lymphocytes [236, 243], endothelial cells [261], and lung fibroblasts [262], and its production in response to pro-inflammatory stimuli suggest the importance of GM-CSF during emergency hematopoiesis. During homeostatic and emergency conditions, GM-CSF

functions to promote the survival, and proliferation of GMPs through STAT5A/B signaling [263] and the differentiation of granulocyte and macrophage colonies from CFU-GM [243, 244]. Studies have also demonstrated the importance of the expression of GM-CSFR on myeloid HPC to maintain myeloid versus lymphoid potential [264]. Furthermore, GM-CSF is chemoattractive to immature and mature neutrophils *in vitro* and *in vivo* [265, 266] and enhances neutrophil anti-microbial functions and promotes neutrophil survival [267] due to the presence of GM-CSFRs on the surface of mature neutrophils. GM-CSF can promote monocytes to differentiate into an inflammatory dendritic cell [268, 269].

As described above in the IL-3 section, studies using βc deficient mouse showed impaired eosinophil development, pulmonary lung disease, an inability to respond to parasite challenge, and HPCs were unable to respond to GM-CSF treatment [241]. Similarly, although double mutant mice deficient in IL-3, IL-5 and GM-CSF signaling pathways displayed reduced eosinophil numbers, hematopoiesis and recovery following challenge with the pathogen *Listeria monocytogenes* was normal [242]. The hematopoietic CSFs that compensate for the loss of GM-CSF and IL-3 in teleosts are not known. These hematopoietic CSFs may also be present in teleosts, which would allow for normal hematopoiesis to occur in the apparent absence of IL-3 and GM-CSF.

2.3.3 Transcription factors

Commitment of LT-HSCs to the myeloid lineage is an intricate regulation of the transcription factors expressed, their relative levels to one another, and their expression on a temporal scale. Transcription factors can act

antagonistically or co-operatively. Thus, the presence or absence of a TF partner, or the relative levels of a TF to its antagonistic counterpart, determine lineage fate decisions. Furthermore, the expression of a transcription factor in an HSC does not exert the same effect as when it is expressed in a committed progenitor cell. The transcriptional regulation of mammalian hematopoiesis/myelopoiesis has been extensively reviewed elsewhere [270-273], and will only be briefly described here for the purpose of putting advances in the teleost model systems into context.

2.3.3.1 MafB

MAFB, a bZIP transcription factor family member, is highly expressed in LT-HSCs, but not in MPPs, CMPs, or GMPs and was recently found to be involved in restricting proliferation and myeloid lineage differentiation of LT-HSCs [2]. MAFB$^{-/-}$ LT-HSCs showed increased proliferative activity and gave rise to large numbers of primarily myeloid progeny in a mouse repopulation assay [2]. The MAFB$^{-/-}$ HSCs had higher proliferative ability and gives rise to greater numbers of myeloid progeny in response to CSF-1 compared to wild type HSCs, *in vitro*. Furthermore, *in vitro* studies demonstrated that treatment of MAFB$^{-/-}$ HSCs with CSF-1 led to the rapid activation of *PU.1* transcription that suggested MAFB must be down-regulated to allow expression of *PU.1* in MMPs [2]. It appears that MAFB plays an important role in antagonizing the expression of PU.1 and the commitment of MMPs to CMPs. Furthermore, MAFB has been shown to bind ETS-1 though its zipper-binding domain and can act to repress erythroid lineage commitment in CMPs [274].

In zebrafish, the *mafb* orthologue has been identified and mRNA was found expressed in the blood forming regions of the developing embryo [275]. However, the role of MAFB in zebrafish HSCs has not yet been assessed.

2.3.3.2 C/EBPs

CCAAT/enhancer binding proteins (C/EBPs) are members of the family of transcription factors that contain a C-terminal basic leucine zipper domain (bZIP) comprised of a basic region involved in DNA binding and a leucine zipper domain involved in protein interactions [276]. Six members of the C/EBP family have been identified in mammals: alpha, beta, gamma, delta, epsilon and zeta [277]. Orthologues of the C/EBP family of transcription factors have been identified in teleosts [278-281], corresponding to C/EBPα, C/EBPβ, C/EBPγ, C/EBPε, and C/EBPδ.

Expressed in HSCs, CMPs and GMPs [282, 283], C/EBPα has been shown to be involved in directing granulocyte cell fate and in the terminal differentiation of neutrophils, along with C/EBPε. Mice deficient in C/EBPα show diminished numbers of CFU-GM, CFU-M, CFU-G, macrophages and neutrophils [284, 285]. The loss of myeloid cells in C/EBPα deficient mice is reflective of the role that CEBPα plays in determining the fate of a CMP to a GMP lineage versus an MEP lineage [286]. C/EBPα is capable of binding to the *PU.1* promoter [285] and up-regulating *PU.1* expression, to dictate a GMP cell fate [285, 287] (see discussion on PU.1 below). The increase in C/EBPα in GMPs has been shown to inhibit monocyte/macrophage differentiation [288] and initiate differentiation along

the granulocyte lineage by regulating *GCSFR*, elastase and myeloperoxidase gene expression [289-291].

The zebrafish CEBPα orthologue showed 66% amino acid identity to human C/EBPα, while the bZIP domains showed 99% amino acid identity [278]. In zebrafish, *cebpa* was expressed in myeloid cells on the surface of the yolk sac during embryogenesis [278]. At 16 hpf, a population of blood cells co-expressed the transcription factors *gata1*, *pu.1* and *cebpa*, and by 22 hpf, the majority of the *cebpa*$^+$ cells co-expressed *pu.1*, however, not all *pu.1*$^+$ cells expressed *cebpa* [292]. Furthermore, *cebpa* was co-expressed with myeloperoxidase (*mpo*), a marker for granulocytes, but *cebpa*$^+$ cells did not always express *mpo* [292]. These three cell sub-populations likely represent distinct junctures in myeloid cell development: erythromyeloid cells, GMPs and committed neutrophils and their precursors, respectively. The expression of *cebpa* with these additional markers mirrors the importance of C/EBPα in the mammalian system in which C/EBPα is important for committment to a myeloid lineage versus an erythroid lineage, to a granulocyte lineage over a macrophage lineage, and in terminal differentiation of neutrophils. An orthologue of *cebpa* was also identified in Japanese flounder and mRNA was observed in the head and posterior kidney, spleen, liver, gill, heart, brain, skin, intraperitoneal cells, and weakly in the intestine, muscle and PBLs [281]. However, expression of *cebpa* in isolated cells populations was not performed.

Two studies have examined the function of CEBPα in zebrafish primitive myelopoiesis. The injection of a deletion mutant of *cebpa* into zebrafish embryos functioned as a dominant-negative mutation and blocked the production of full-

length CEBPα. These embryos exhibited an increase in *gata1*$^{+}$ expression in the posterior lateral plate mesoderm at 22 hpf and in the intermediate cell mass at 26 hpf, reflective of an erythroid progenitor cell expansion. This expression corresponded to a subsequent increase in circulating erythrocytes based on the increase in *α-hemoglobin* expression, indicative of erythrocytes [292]. However, the expressions of the myeloid specific genes, *mpo* and *l-plastin*, were normal [292]. Based on the pattern of expression, it was suggested that PU.1 acts upstream or in parallel with C/EBPα during zebrafish primitive myelopoiesis [292]. Recently, it has been shown that the sumoylation (a post-translational protein modification) of zebrafish CEBPα inhibited CEBPα transcriptional activity and its ability to interact with and repress GATA1, thus driving lineage commitment of a myelo-erythroid progenitor to that of the erythroid lineage [293]. Taken together, these studies demonstrate the conserved role of CEBPα in the commitment of a CMP to a GMP. However, due to the toxicity of *cebpa* morpholinos to zebrafish embryos, knockdown experiments could not be performed.

Cebpb was identified in rainbow trout as a single intron-less gene and the predicted CEBPβ protein showed 30-34% amino acid identity to mammalian C/EBPβ [279]. The *cebpb* mRNA was detected in the head and posterior kidney, spleen, liver, gill, intestine, muscle and PBLs [279]. Japanese flounder CEBPβ also showed a low (33-38%) amino acid identity to other vertebrate sequences, but retained 95% amino acid identity in the bZIP domain. The *cebpb* mRNA was expressed in the head and posterior kidney, liver, gill, brain, peritoneal cavity fluid and PBLs, with low mRNA levels in the heart, intestine, mucus, eye and spleen

[280]. In zebrafish, CEBPβ showed 49% amino acid identity to human C/EBPβ and *cebpb mRNA* was detected in cells on the surface of the yolk sac, corresponding to the myeloid cells that normally spread over the yolk sac early in embryogenesis [278]. A *cebpb* transcript was also identified in a differential cross-screen of goldfish proliferative phase and senescence phase primary kidney macrophages (PKMs), and was up-regulated in goldfish monocytes, and expressed in low levels in progenitors and macrophages [294]. However, the functional role of CEBPβ has not been examined in teleost myelopoiesis.

The orthologues of C/EBPδ, C/EBPγ and C/EBPε exist in teleosts. The *cebpd* and *cebpg* transcripts were identified in zebrafish and show a ubiquitous expression pattern in embryos [278]. CEBPδ and CEBPγ showed 57 and 50% identity to their human counterparts on the amino acid level. However, their bZIP domains showed higher conservation to their human counterparts, with 86% and 76% amino acid identity, respectively [278]. The *cebpe* orthologue was identified in Japanese flounder and its corresponding predicted protein had a 27% overall amino acid identity and a 90% amino acid identity in the bZIP domain compared to the mammalian counterparts, but failed to cluster with other *cebpe* sequences in phylogenetic analysis [280]. The *cebpe* mRNA was detected in the head and posterior kidney, spleen, brain, peritoneal cavity fluid and at low levels in the PBLs. However, the functional role of these C/EBPs in teleost myelopoiesis is unknown.

2.3.3.3 PU.1

The Ets transcription family member PU.1 is well known as the master transcriptional regulator of mammalian myelopoiesis through an antagonistic relationship with GATA1, recently reviewed by [295]. At the N-terminus, PU.1 comprises of an acidic domain and a glutamine rich domain that are involved in activation of transcription, and a PEST domain important for protein interactions [295]. At the C-terminus, PU.1 has an Ets domain important for binding the DNA consensus sequence AAAG(A/C/G)GGAAG [296]. Mice deficient in PU.1 (*PU.1*$^{-/-}$) have reduced CLPs, and GMPs, increased numbers of MEPs, and lack B-cells, T-cells, monocytes/macrophages as well as severely reduced numbers of granulocytes [297-301]. *PU.1* is expressed in HSCs, CLPs and at varying levels in CMPs, increasing as these progenitors are induced to differentiate into monocytes/macrophages and neutrophils [302]. At the CMP stage, PU.1 antagonizes with GATA1 to determine whether the CMP commits to a GMP or a MEP. PU.1 binds to GATA1 and inhibits GATA1 from binding to and initiating transcriptional activation of a number of erythroid genes that are important for commitment to an erythroid lineage [295, 303, 304]. The reverse is also true; GATA1 can bind to PU.1 and inhibit the binding of PU.1 and transcriptional activation of a number of myeloid genes [295, 303, 304], including to the promoters of *CSF-1R* [305-307] and *GCSFR* genes [291, 307, 308]. Therefore, the lineage fate decision along a GMP or a MEP fate is a balancing act in timing and relative protein levels of PU.1 and GATA1.

PU.1 also plays a role at the GMP stage to regulate commitment to a granulocyte or macrophage lineage. Increased levels of PU.1 at the GMP stage,

along with AP-1 association, drives a monocyte cell fate, while lower levels of PU.1 drives granulocyte cell fate [285, 287]. Furthermore, PU.1 induces *EGR-2* and *NAB-2* expression [287]. The EGR-2/NAB-2 transcription factors function to repress neutrophil genes by antagonizing GFI1, an important transcription factor in the initiation of neutrophil differentiation [287], discussed in section 1.4.3.

An orthologue of PU.1 has been identified in teleosts. In the Japanese flounder, *pu.1* mRNA was detected in the head and posterior kidney, spleen, heart, PBLs, intraperitoneal cells, and weakly in the intestine and gill, but was absent from the liver, skin, muscle and brain [281]. In zebrafish, *pu.1* was identified as a single gene copy and analysis of the predicted protein sequence showed the conserved transactivation, PEST, and DNA-binding domains. Although the overall amino acid identity to other PU.1 proteins was 48-53%, the DNA-binding domain of zebrafish PU.1 showed 83% amino acid identity to mammalian PU.1 [309]. Examination of the zebrafish *pu.1* promoter region predicted potential binding sites for PU.1 and CEBPα [310]. The expression of *pu.1* is first detected at 12 hpf in blood cells from the posterior lateral plate mesoderm, later in the intermediate cell mass, and finally in the kidney, and these *pu.1*$^+$ blood cells give rise to myeloid cells [309-311]. The population of *pu.1*$^+$ cells represents myeloid HPCs, myeloid precursors, monocytes/macrophages and neutrophils during both primitive and definitive myelopoiesis in the zebrafish [14, 311].

Knockdown of *pu.1* in zebrafish using morpholinos showed a large reduction in the number of cells positive for *mpo* and *l-plastin* mRNA, markers of granulocytes and monocytes/macrophages [312, 313]. In addition to the loss of myeloid cells, an increase in *gata1* expression was observed, and these *gata1*$^+$

cells gave rise to mature erythrocytes [312]. Conversely, *gata1* morphants failed to develop mature erythrocytes and showed an increase in the number of *pu.1*$^{+}$, *mpo*$^{+}$ and *l-plastin*$^{+}$ cells [312, 313]. Ectopic expression of *pu.1* or *gata1* was observed in *gata1* or *pu.1* morphants, respectively, suggesting the conversion of progenitors to an alternate lineage [312, 313]. Microarray analysis of genes regulated by PU.1 revealed the regulation of ~250 genes, including *cebpa*, *csf-1r* and myeloid-specific peroxidase *(mpx)*, among others [314]. Taken together, PU.1 has a conserved role in dictating a myeloid lineage, opposing GATA1 and the transcriptional activation of erythroid genes.

A pu.1-like gene (spi-1 like, *spi-1l*) was also identified in zebrafish. The predicted amino acid sequence of SPI-1l showed 45% amino acid identity to zebrafish PU.1, and retained all three domains [315]. *In situ* hybridization revealed a population of blood cells positive for *pu.1* and *spi-1l*, in addition to a population of single positive *pu.1* cells [315]. However, only a few single-positive *spi-1l* cells were observed. *Spi-1l* morphants showed a loss of *mpx* and *l-plastin* positive cells, indicative of a loss in granulocytes and monocytes/macrophages [315]. Unlike *pu.1* morphants, no change in *gata1* expression was observed, suggesting that SPI-1l acts downstream of PU.1, and plays an important role in myeloid cell differentiation [315].

2.4 Commitment of bi-potent myeloid progenitors to the macrophage or neutrophil lineage

2.4.1 Macrophage development

2.4.1.1 Progression of cell development

In mammalian systems, the progression of macrophage development proceeds from a committed macrophage progenitor, monoblast, promonocyte, monocyte and then to a mature tissue macrophage, reviewed by [316-318]. While the presence of a unipotent committed macrophage progenitor has yet to be unequivocally demonstrated in the teleost system, progenitor/precursor cells that give rise to monocytes and macrophages have been demonstrated. *In vitro*, a spontaneous proliferating trout RTS-11 cell line has two predominant cell types; a round non-adherent cell type that appears to be a pre-monocyte or myeloid precursor and an adherent macrophage-like cell, arising from the non-adherent cell type [319]. The cultivation of trout kidney progenitor-like cells developed a trout primary kidney monocyte culture that contained progenitor cells, promonocyte-like cells, and monocytes [320]. Furthermore, the generation of goldfish primary kidney macrophage cultures demonstrated that small mononuclear cells became monocytes and mature macrophages, *in vitro* (discussed in the goldfish macrophage culture system section of the thesis). In the zebrafish model system, whole kidney marrow was added to a kidney fibroblast layer and was shown to maintain HPCs and precursor cells that then differentiated into myeloid and lymphoid cells [321]. Recently, the development of a zebrafish methylcellulose colony forming unit assay suggested the presence of a common erythro-myeloid HPC [322]. *In vivo* studies, primarily in the zebrafish,

have demonstrated that monocytes/ macrophages arise from the hematopoietic organ [36, 323-326], migrate to various tissues [327], and both primitive and definitive macrophages are motile, migrate to the site of insult, and readily phagocytose particles or pathogens [13, 36, 328-330]. The identification of progenitor cells that are capable of differentiating into monocytes and macrophages suggests a conserved macrophage differentiation pathway in vertebrates.

2.4.1.2 Receptors and growth factors

2.4.1.2.1 Colony-stimulating factor-1 (CSF-1)

The central growth factor that regulates the survival, proliferation, and differentiation of macrophages and their precursors is colony-stimulating factor-1 (CSF-1) [331-334]. The structure, function and regulation of mammalian CSF-1 has been extensively reviewed elsewhere, and therefore, will only be briefly described here [216, 332, 335-337]. CSF-1 has been identified in human, mouse, rat [338], bovid [339], avian [340], and teleost [341, 342] species. The *CSF-1* gene is comprised of 10 exons [343] and is found on human chromosome 1p13-21 [344, 345], and mouse chromosome 3 [346, 347]. Alternative splicing of the *CSF-1* gene can lead to the production of transcripts ranging from 1.6-4.5 kbp [343] and produce a secreted glycoprotein, a secreted proteoglycan, or a membrane-bound glycoprotein that can be proteolytically cleaved from the surface, reviewed by [216, 332]. However, only the first 149-150 aa of the N-terminal portion of the CSF-1 core protein has shown to be important for biological function [335, 343]. Structural analysis of the CSF-1 protein showed

two monomers, each bundles of four α-helices, are covalently linked by an interchain disulphide bond to form a dimer [348].

During homeostasis, soluble CSF-1 can be detected in the human blood around 2.4 ng/mL [349]. CSF-1 is produced by an array of cell types including fibroblasts, endothelial cells, and bone marrow stromal cells, reviewed by [216]. In addition, activated T-cells [350-352], monocytes, macrophages [353, 354], fibroblasts, and endothelial cells [216] can produce CSF-1. CSF-1 production by activated cell types suggests a role for CSF-1 at the site of inflammation, which may be necessary for the rapid recruitment, differentiation and activation of macrophages and their precursors.

2.4.1.2.2 Interleukin-34

Recently, IL-34 was identified as another growth factor involved in mediating macrophage development in mammals, in addition to CSF-1 [355-357]. *IL-34* gene was first identified in humans on chromosome 16q22 and orthologues of *IL-34* were identified in chimpanzee, mouse, rat [355] and avian (chicken and zebra finch) species [340]. The 726 bp mRNA open reading frame corresponds to a 241 aa, 39 kDa human IL-34 protein that does not show homology to any other human protein and or contain any known conserved structural motifs [355]. A survey of *IL-34* mRNA in tissues showed broad distribution; *IL-34* mRNA was observed in the kidney, liver, thymus, heart, brain, lung, small intestine, colon, prostate, testis, and ovary, with the highest level of expression occurring in the spleen [355].

The IL-34 monomers associate into homodimers, and bind to colony stimulating factor-1 receptor (CSF-1R). Interestingly, human IL-34 binds to CSF-1R with a higher affinity (Kd 1 pM) compared to that of CSF-1 (Kd 34 pM) [355]. However, this is not the case for murine IL-34 and murine CSF-1, in which murine CSF-1 was shown to have higher affinity than murine IL-34 for the CSF-1R [356]. The hierarchy in binding of the CSF-1R ligands may provide a mechanism for differential signaling depending on the bound ligand. Structural analysis in the avian model predict different binding sites of CSF-1 and IL-34 on CSF-1R, also providing a means for affecting differential signaling in response to the ligands [340]. Binding of IL-34 to CSF-1R on monocytes lead to phosphorylation of ERK1/2, with similar kinetics to CSF-1, and induced CFU-M from bone marrow cells, and stimulated monocyte survival/proliferation [355, 356].

IL-34 has demonstrated functions outside of hematopoiesis. IL-34 in conjunction with receptor activator of NF-kB ligand (RANKL) acts to promote the differentiation of osteoclasts from splenocytes and bone marrow [358], and may play a role in regulating bone reabsorption. Additionally, IL-34 may be involved in the development of brain microglial cells that are dependent on CSF-1R signaling [356, 359].

2.4.1.2.3 Colony-stimulating factor receptor

The *CSF-1R* has been identified in human, murine, rat, cat, dog, avian [340], and teleost species (see teleost CSF-1R section below). The *CSF-1R* gene, shown to map to the proto-oncogene *c-fms*, is found on chromosome

5q33.3 in humans [360, 361] and chromosome 18 in mice [362] and contains 22 exons [363]. As a member of the type III tyrosine kinase family of receptors, the CSF-1R protein structure is composed of a signal peptide, five immunoglobulin domains (D1-D5) with 10 conserved cysteine residues and a number of predicted N-linked glycosylation sites to comprise the extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain that is interrupted by a intervening sequence of ~70 aa [364, 365], reviewed by [366].

The binding of homodimeric CSF-1 to the first three immunoglobulin domains (D1-D3) of a single CSF-1R protein initiates the association of the D4/D5 domains of the bound CSF-1R with the D4/D5 domains of an unbound CSF-1R, and in the process, completes the homodimerization of CSF-1R and triggers receptor activation [367]. The concurrent completion of ligand binding and activation triggers autophosphorylation of the intracellular tyrosine residues and activation of JAK/STAT, PI3K/Akt, and MAPK pathways, as well as pathways for receptor-mediated internalization and destruction, reviewed by [273, 366, 368].

Within the hematopoietic system, CSF-1R protein is primarily found on macrophages and their precursors and has been used as a marker of cells along the macrophage lineage in mammalian systems [333, 365]. CSF-1R progressively increased with macrophage differentiation [216]. However, *CSF-1R* mRNA transcripts have been observed in neutrophils [369], but is not translated into protein or expressed on the surface of neutrophils.

2.4.1.2.4 Biological functions of colony stimulating factor-1

In addition to the regulation of survival, proliferation, and differentiation of macrophages and their precursors [331-334], CSF-1 has been shown to exert pro-inflammatory effects on monocytes and macrophages. These effects include the enhancement of macrophage chemotaxis, phagocytosis of pathogens, and the production of antimicrobial agents, reviewed by [273, 366]. CSF-1 is a pleiotropic cytokine and functions in a number of other biological systems outside of hematopoiesis as exemplified by the osteopetrotic (*CSF1*$^{op/op}$/ *CSF1*$^{op/op}$) mouse which produces a truncated, non-functional CSF-1 protein [370]. The *CSF1*$^{op/op}$/ *CSF1*$^{op/op}$ mice display a drastic reduction in macrophage and osteoclast numbers, leading to defects in bone remodeling and osteopetrosis, a lack of teeth and defects in fertility and breast development [371-373]. Furthermore, the disruption of the *CSF-1R* gene, (*CSF-1R*$^{-/-}$) in mice showed similar, although more severe, defects to that of the *CSF1*$^{op/op}$/ *CSF1*$^{op/op}$ mouse [374], likely due to the absence of all signaling through the CSF-1R. Recent studies have shown that induced expression of *IL-34* in *CSF1*$^{op/op}$/ *CSF1*$^{op/op}$ mice can partially rescue the phenotype [356], suggesting functional redundancy between CSF-1 and IL-34.

2.4.1.2.5 Teleost colony stimulating factor-1

Teleost *csf-1* (*mcsf*) was first identified in the goldfish as a 600 bp mRNA transcript that was present at high levels in spleen tissue, monocytes, and phorbol ester-activated monocytes [341]. The *csf-1* transcript encoded for a 199 aa precursor protein, with the mature CSF-1 protein predicted to have a molecular weight of 22 kDa. The goldfish CSF-1 has 27% aa identity to human

CSF-1 [341]. Alignment of goldfish CSF-1 with mammalian CSF-1s showed conservation of four cysteine residues required for protein folding, similar to that of mammalian CSF-1 [341]. Ligand-receptor binding studies demonstrated that homodimeric CSF-1 could bind to soluble CSF-1R (see teleost CSF-1R section below). Functional characterization of a recombinant goldfish CSF-1 was shown to induce monocyte proliferation and differentiation, which was abrogated in the presence of sCSF-1R or in monocytes transfected with *csf-1r* RNAi oligos, [341, 375]. Recombinant goldfish CSF-1 also aided in the long-term survival of mature macrophages *in vitro* [375]. The recombinant CSF-1 protein was chemoattractive to PKMs, and promoted their ability to perform phagocytosis and produce antimicrobial compounds [376], suggesting a pro-inflammatory role for CSF-1 in goldfish.

Two *csf-1* genes were later identified in trout and zebrafish, termed *mcsf-1* and *mcsf-2*, and a second goldfish *mcsf* transcript was identified [342]. The trout and zebrafish *mcsf-1* genes encoded for proteins of 593 and 526 aa, the trout and zebrafish *mcsf-2* genes encoded for proteins of 276 and 284 aa, respectively, while the goldfish *mcsf* gene encoded for a 544 aa protein [342]. All of the identified transcripts possessed a signal peptide, a CSF-1 domain, a transmembrane domain, and a short cytoplasmic domain [342]. However, the N-terminal region of all teleost CSF-1 proteins showed high homology (46-88%), consistent with the important role of the CSF-1 N-terminal portion for biological function.

The genomic structure of the identified *mcsf*s also differed. The zebrafish *mcsf-1*, found on chromosome 11, possessed seven exons and *mcsf-2*, found on chromosome 8, possessed nine exons. Based on syntenic analysis, the two *mcsf*

genes appeared to have arose through a chromosomal or genome duplication [342]. Examination of the intron-exon structure of trout *mcsf*s showed *mcsf-1* to possess 10 exons and 9 introns, and *mcsf-2* to have 9 exons and 8 introns [342].

Along with differing genomic organizations, trout *mcsf-1/-2* are differentially expressed in tissues. The *mcsf-1* transcript was predominantly expressed in the spleen, intestine and brain, while *mcsf-2* was predominantly expressed in the head kidney, gills, muscle and liver [342]. While a recombinant trout MCSF-1 protein was produced and demonstrated to induce the proliferation of head kidney macrophages, a recombinant trout MCSF-2 protein was not produced to examine whether there was differential regulation of macrophage function by the MCSFs [342]. Whether MCSF-1 and MCSF-2 are functionally redundant or functionally partitioned (sub-functionalization), remains to be determined.

2.4.1.2.6 Teleost colony stimulating factor-1 receptor

The *csf-1r* sequences have been identified in a number of teleost species including puffer fish [377, 378], zebrafish [379], rainbow trout [380], gilthead seabream [381] and goldfish [382]. CSF-1R protein appears to be a marker of monocytes and macrophages in teleosts [381-383]. Analysis of the puffer fish *csf-1r* gene shows a 21 exon gene structure in fish, same as in mammals. However, the puffer fish *csf-1r* gene only spans 10.5 kbp versus the mammalian 55 kbp, due to the decrease in the size of the intronic sequences [377]. The *csf-1r* mRNA open reading frame encodes for a 975 aa protein, with a signal peptide, an extracellular domain with 10 conserved cysteine residues characteristic of

immunoglobulin domains, transmembrane domain, and an intracellular kinase domain with an interruption of 70 bp [377]. While CSF-1R of puffer fish is only 39% similar to human CSF-1R, the kinase domain is considerably more conserved, particularly in the motifs associated with signaling. The fish *csf-1r* gene was linked with *pdgfrb-1* [377].

A second *csf-1r* gene (*csf-1r-2*) was also identified in puffer fish, and linked with a second *pdgfrb* (*pdgfrb-2*). The *csf-1r-2* gene was comprised of 22 exons and had a different intron-exon organization than *csf-1r-1* [378]. Despite the similar protein structure of the two CSF-1Rs, the amino acid sequences were only 39% identical. The *csf-1r* mRNAs were differentially expressed in tissues. The *csf-1r-1* was expressed in blood, brain, eye, gill, heart, kidney, ovary, skin, and spleen, while *csf-1r-2* was expressed in the blood, brain, eye, gill, heart, kidney, liver, muscle, skin, spleen and testis. [378].

The duplication of *csf-1r* genes was also observed in cichlids, the green-spotted pufferfish, medaka, and *Tetraodon* (found on chromosomes 1 and 7), with the *csf-1r-2* duplicated genes appearing to have undergone evolutionary selection or diversification while the *csf-1r-1* gene appeared to resemble that of the ancestral gene [384]. It was proposed that the fish specific whole genome duplication generated the two paralogues of *csf-1r* in fish, as well as two *pdgfrb* and *kit* genes, and that *kit* and *csf-1r-2* may have been retained to play a role in the survival, migration and differentiation of melanocytes and xanthophores, important pigment cells involved in fish coloration patterns [384].

The *panther* (*fms*) mutant zebrafish have a defect in the *csf-1r* gene, and mutant fish fail to develop their characteristic pigment pattern of black and yellow stripes. The CSF-1R was found to be important in the survival, migration and

differentiation of precursors to yellow xanthophores in zebrafish [385, 386]. However, unlike that of the CSF-1R$^{-/-}$ mice, there were no reports of hematopoietic defects in *panther* zebrafish. The lack of hematopoietic defects may be due to the presence of another *csf-1r* gene, a low level of *csf-1r* expression, or a differential requirement for CSF-1R during embryonic macrophage development versus adult macrophage development in teleosts. However, CSF-1R was shown to be important in the migration of primitive macrophages to tissues, such as the brain, retina and epidermis upon comparing primitive macrophage distribution and migration in wild-type and *panther* zebrafish [379]. Furthermore, *csf-1r* mRNA was detected in inflammatory macrophages from 3 dpf zebrafish embryos [330]. Taken together, these results support a role for CSF-1R in teleost macrophage biology.

A full-length *csf-1r* cDNA sequence was identified in trout, with an open reading frame of 2904 bp encoding for a 967 aa protein, predicted to be ~109 kDa. Trout CSF-1R had 40% aa identity to that of human and mouse, and 54% and 52% identity to that of puffer fish and zebrafish CSF-1R [380]. The trout *csf-1r* gene was similar to that of the ancestral gene, and mRNA was found in the head-kidney, spleen, blood, ovary, and showed lower mRNA levels in the liver, brain, heart, muscle, gill, and skin [380]. Southern blotting revealed two bands in each lane, suggestive of a second *csf-1r* gene in trout. However, a second *csf-1r* gene was never identified.

CSF-1R was also identified in goldfish as a 975 aa integral membrane bound protein (mCSF-1R) that possessed the five Ig extracellular domains with multiple N-linked glycosylation sites, a transmembrane domain, and an intracellular tyrosine kinase domain [382]. The mRNA of mCSF-1R could be

detected in progenitor, monocyte and macrophage subpopulations, and an antibody produced against the first two Ig domains of CSF-1R was able to recognize monocytes and macrophages [382]. However, unlike mammalian neutrophils, zebrafish and goldfish neutrophils do not appear to express mRNA for *csf-1r* [330, 387]. Additionally, alternative splicing of the *csf-1r* transcript encoded for a soluble form of the CSF-1R (sCSF-1R), possessing only the D1 and D2 Ig domains, important for binding of CSF-1. The s*csf-1r* mRNA was expressed by leukocytes within the progenitor and macrophage populations, but not in the monocyte subpopulation [382]. Furthermore, addition of a recombinant purified sCSF-1R dampened the proliferation of spontaneously growing and differentiating PKMs [382]. The increased production of the sCSF-1R by PKMs during senescence phase suggested that sCSF-1R was involved in the negative regulation of CSF-1 signaling through mCSF-1R [341, 382].

2.4.2 Neutrophil development

2.4.2.1 Progression of cell development

Following the commitment of the CFU-GM to a committed granulocyte progenitor cell, terminal differentiation through a promyelocyte, myelocyte, and metamyelocyte stages occur to give rise to a mature neutrophil, and is regulated through growth factor and transcription factor signaling, reviewed by [388]. Similar to that of mammals, the differentiation of fish neutrophils appears to occur through various stages, based on morphological and cytochemical characteristics, and include the promyelocyte, myelocyte, metamyelocyte and the mature neutrophil, which sometimes had a segmented nucleus [36, 323, 324,

326, 389]. These neutrophils were shown to migrate from the hematopoietic organ to the site of wounding, pathogen injection, or transformed cell injection [14, 36, 390], in response to a hydrogen peroxide attractant produced by cells at the site of damage [328]. However, the responding neutrophils had low phagocytic activity [14], or engulfed small fragments of the pathogen [328]. *In vitro*, treatment of zebrafish kidney marrow cells with G-CSF gave rise to CFU-GM in a methylcellulose assay [322]. However, there is a lack of *in vitro* culture systems for studying progenitor cell to neutrophil differentiation. The identification of functional neutrophils and their precursors, suggests the presence of a committed granulocyte progenitor cell in teleosts.

2.4.2.2 Receptors and growth factors

2.4.2.2.1 Granulocyte colony-stimulating factor

Neutrophils contribute to both innate and adaptive immune responses. They are capable of chemotaxis, phagocytosis, the production of antimicrobial molecules, and the formation of extracellular traps [391-396]. Furthermore, upon activation, neutrophils can produce a number of chemokines, pro-inflammatory and anti-inflammatory cytokines, as well as the colony-stimulating factors G-CSF, CSF-1, GM-CSF, IL3 and SCF, reviewed by [397, 398]. However, neutrophils are short lived with estimates ranging from 6-90 hrs. Thus, neutrophils need to be continuously replaced. G-CSF is the primary CSF that mediates the proliferation, differentiation, survival and activation of neutrophils and their progenitors, and has been reviewed extensively by [216, 399].

The *GCSF* gene has been identified in human [400], mouse [401], rat [402], cat [403], bovine [404], ovine [405], porcine [406], avian [407-410], and fish species [409, 411, 412]. The gene is located on human chromosome [400], and on mouse chromosome 11. Human *GCSF* genomic organization consists of 5 exons and 4 introns and two alternatively spliced transcripts can be generated [413, 414]. The predominant, more biologically active transcript encodes for a 174 aa mature protein, identified as a member of the class I cytokines, while the second transcript produces a 177 aa mature protein, both with a predicted molecular weight of 19 kDa [413, 414]. There are no predicted N-linked glycosylation sites on either variant [413, 414]. The mouse *GCSF* gene organization is 5 exons and 4 introns, and spans ~ 2.7 kbp [415], like that of the human *GCSF* gene. Transcription of this gene produces a 1.5 kbp transcript with an open reading frame of 624 bp, and a 3' UTR with AU rich regions [415]. The 178 aa mature protein [415, 416] has a predicted size of 19 kDa, but is found at a molecular weight of 24-25 kDa from mouse lung-conditioned media [401]. The mouse GCSF protein did not posses any predicted N-linked glycosylation sites, and instead the increase in observed molecular weight may be due to O-linked glycosylation [416]. Unlike human *GCSF*, no alternative splice variants were found for mouse *GCSF* [415]. In humans, the normal GCSF concentration in blood ranges from 30-162 pg/mL, and can be massively up-regulated during infection up to 3200 pg/mL [417-419].

The transcription of *GCSF* is controlled by an upstream promoter region that has a tumor necrosis factor alpha response region that has been shown to be bound by NF-kB p65 and NF-IL6, reviewed elsewhere by [216, 420]. Furthermore, GCSF is produced by activated monocytes/macrophages,

neutrophils, fibroblasts and endothelial cells in response to a number of pro-inflammatory stimuli, reviewed elsewhere by [216, 399, 420].

2.4.2.2.2 Granulocyte colony-stimulating factor receptor

The gene for granulocyte colony-stimulating factor receptor (*GCSFR*, CD114) has been identified in human [421], mouse [308], bovine [422], and teleosts [412, 423]. The *GCSFR* gene is found on human chromosome 1p35-34.3 [421] and on mouse chromosome 4 [308]. Both the human and mouse *GCSFR* genes have a 17 exon/16 intron structure [308, 424], however, the mouse also possesses a second *GCSFR* pseudogene [308]. The protein structure of GCSFR is comprised of a signal peptide, an immunoglobulin-like domain, a cytokine receptor homology (CRH) domain containing the class I cytokine receptor superfamily motif W-S-X-W-S, three fibronectin domains, a transmembrane domain, and an intracellular cytoplasmic signaling domain containing three motifs termed Box 1, Box 2, and Box 3, important for signal transduction [399, 425]. Based on their protein structure and conserved motifs, the human and mouse integral membrane GCSFR proteins were placed in the type I cytokine receptor family.

While there are reports of GCSFR on other hematopoietic cells such as monocytes [426] and lymphocytes, as well as some non-hematopoietic cells, GCSFR is primarily found on neutrophils and their precursors [399, 427]. Neutrophils up-regulate their levels of GCSFR as they differentiate from a progenitor cell to a mature neutrophil, with 50-500 GCSF receptors per cell [428]. Structural analysis has demonstrated that GCSF forms a homodimer, binding two

GCSFRs, leading to their homodimerization in a 2:2 complex [429-431]. However, 1:1 and 4:4 complexes of GCSF/GCSFR have also been documented [432, 433], suggesting that the association of GCSF with GCSFR may be dependent on the availability or abundance of GCSF present. Binding of a homodimeric GCSF complex to the Ig and CRH domains [429-431] of two GCSF receptors triggers intracellular signaling through the JAK/STAT, Ras/Raf/Erk, or PI3K pathways [425, 427, 434]. These signaling pathways ultimately lead to the migration, survival, proliferation, and differentiation of neutrophils.

Control of GCSFR signaling in neutrophils is modulated through transcriptional activation of the *GCSFR*, the production of a soluble receptor, and cleavage of surface GCSFR. The upstream promoter region of the *GCSFR* gene has sites binding for the AP-1, AP-2, C/EBPα, NF-IL6, GATA-1, and PU.1/SPI1 transcription factors [291, 308]. These transcription factors are involved in myeloid cell commitment and the regulation of the *GCSFR* expression during neutrophil development. The *GCSFR* expression is also modulated at the post-transcriptional level, with the production of a soluble GCSFR that lacks a transmembrane region [425] and may be involved in preventing binding of GCSF to membrane GCSFR. Lastly, the GCSFR signaling can be controlled at the protein level. The release of elastase by neutrophils has been shown to cleave the GCSFR extracellular domain from the surface of neutrophils, and this cleavage of surface GCSFR prevents GCSF induced granulopoiesis *in vitro* [435].

2.4.2.2.3 Biological activity of granulocyte colony stimulating factor

The targeted gene disruption of *GCSF* and *GCSFR* has demonstrated the important functional roles of GCSF *in vivo*. The GCSF deficient mice showed severe neutropenia (70% reduction in circulating neutrophils), reduction in monocyte and macrophage numbers, and ~50% reduction in the numbers of neutrophil precursors present in the bone marrow [436, 437]. However, this phenotype could be reversed with daily injections of GCSF. Furthermore, GCSF deficient mice infected with *Listeria monocytogenes* were unable to control bacterial replication, resulting in 50% mortality by day five of infection, compared to the lack of mortality in wild-type mice [436, 437]. The disruption of *GCSFR* in mice lead to a similar phenotype, However, a further reduction in circulating neutrophil number (88% reduction), a decrease in the number of neutrophils in the spleen, and in the number of myeloid progenitors in the bone marrow was observed due to the involvement of GCSFR signaling in the production/maintenance of CFU-GM and CFU-G [434, 438]. Furthermore, the neutrophils from GCSFR deficient mice showed increased apoptosis compared to neutrophils from wild-type mice, demonstrating the role of GCSFR mediated signaling in neutrophil survival [438]. Even in mice deficient for GCSF, GM-CSF, and CSF-1, a low level of circulating neutrophils are observed, although the triple growth factor deficient mice are exceptionally prone to infection [439]. It is interesting to note that GCSF deficient mice did not display a complete lack of neutrophils, similar to the situation described previously in CSF-1 deficient mice. This may suggest the presence of a second, unidentified ligand of GCSFR, in

addition to a GCSFR-independent neutrophil development pathway regulated in part by IL-6 and GM-CSF [440, 441].

GCSF treatment of bone marrow cells, *in vitro*, induced CFU activity where mainly neutrophil colonies were produced, with a few macrophage and eosinophil colonies [416]. GCSF has been shown to promote the proliferation of neutrophil precursors by shortening their cell cycle time, and increasing the number of neutrophil precursors that accumulate in the bone marrow, reviewed by [399]. The release of mature neutrophils, their terminal differentiation, survival, and activation, is also mediated by GCSF *in vitro* and *in vivo*, reviewed by [399]. Lastly, GCSF has been used in the clinical setting to increase peripheral blood neutrophil numbers for treatment of disease and for stem cell mobilization from the bone marrow into the peripheral blood, reviewed by [442, 443].

2.4.2.2.4 Teleost granulocyte colony-stimulating factor

The teleost granulocyte colony-stimulating factor (*gcsf*) gene was first identified in Japanese flounder, fugu, and the green-spotted pufferfish [409]. Both the fugu and green-spotted pufferfish have two *gcsf* genes, termed *gcsf-1* and *gcsf-2*, while only an orthologue of *gcsf-2* was identified in flounder [409]. Phylogenetic analysis of vertebrate *gcsf*s predicted fish *gcsf-1* to be the ancestral gene, while *gcsf-2* was predicted to be the duplicated gene. Alignment of the fish GCSFs with human and mouse GCSF showed low identity, ranging from no significant identity to 34% amino acid identity [409]. Despite the low amino acid identity of fish to mammalian GCSF, all fish *gcsf* genes retained a 5 exon/ 4 intron structure with a conserved tumor necrosis factor alpha response element

in the promoter region. Furthermore, the predicted transcripts have an open reading frame of 561-636 bp, corresponding to a predicted protein of 20-23 kDa, and 4-5 AU rich sequences in their 3' UTRs shown to be involved in mRNA instability and degradation [409]. Determination of the ratio of synonymous to asynonymous nucleotide substitutions (Ks/Ka) in fish *gcsf* genes ranged from 0.467 to 0.961 with an average of 0.793, demonstrating that positive selection was occurring in GCSFs of fish (and chicken) [409]. Two *gcsf* genes were also identified in the black rockfish (*Sebastes schlegelii*) [411] and in zebrafish [412] (O. Svoboda and P. Bartunek, personal communication), while only one *gcsf* gene has been identified in trout (NM_001195184).

Flounder *gcsf-2* mRNA levels were highest in the spleen, kidney, and gill. However, *gcsf-2* mRNA was still detected in the brain, eyes, heart, peripheral blood leukocytes, ovary, skin, and stomach, but was not detected in intestine, liver, or muscle tissue [409]. As expected, *gcsf-2* mRNA levels were up-regulated in kidney and peripheral blood leukocytes following treatment with lipopolysaccharide (LPS) or a mixture of concanavalin A and phorbol esters (ConA/PMA) [409]. The black rockfish *gcsf-1* showed expression in the peripheral blood leukocytes, spleen, gill, intestine and muscle [411]. However, black rockfish *gcsf-2* was ubiquitously expressed in the peripheral blood leukocytes, head and trunk kidney, spleen, gill, intestine, muscle, liver and brain [411]. Although both *gcsf-1* and *gcsf-2* black rockfish mRNA levels were upregulated in PBLs treated with LPS or ConA/PMA, differential kinetics and levels of expression were observed between the two *gcsfs* [411]. It appears that *gcsf-1* may be rapidly induced with sustained levels following stimulation, whereas *gcsf-2* is only slightly upregulated and showed a drastic increase in mRNA levels after ConA/PMA

treatment for 24 hrs [411]. Taken together, these data suggest that GCSF-1 may play an important role during inflammation, although functional studies are required to determine the roles of GCSF-1 and GCSF-2 in teleost granulopoiesis and inflammation.

Functional studies on fish GCSF-1 are limited. Only two manuscripts report on the function of GCSF-1 and both utilize the zebrafish model system. *In vitro*, precursor cells from whole kidney marrow were sorted, plated in a methylcellulose colony forming unit assay and treated with either GCSF or a combination of GCSF and erythropoietin (EPO). While both treatments led to CFUs containing granulocytes and macrophages, based on cell morphology and expression of myeloid genes, the combination of GCSF and EPO also supported the formation of erythroid CFUs [322]. *In vivo*, morpholino mediated knockdown of *gcsfr* in zebrafish showed a decrease in numbers and migration of cells expressing both neutrophil and macrophage specific transcripts, during both primitive and definitive hematopoiesis in the zebrafish embryo. However, a population of myeloid cells remained, despite morpholino mediated knockdown of *gcsfr*, suggesting the presence of a GCSFR-independent pathway of myeloid cell development and migration [412]. Injection of wild-type zebrafish with *gcsf* mRNA increased the number of myeloid and *gcsfr*$^{+}$ cells, while injection of *gcsf* mRNA into *gcsfr* morpholino zebrafish did not result in an increase in myeloid cell numbers [412]. These studies suggested GCSF-1 participates in myeloid cell development, similar to that observed in mammalian systems. No functional studies have been performed using GCSF-2, and the role(s) of GCSF-2 in myelopoiesis remain to be elucidated.

2.4.2.2.5 Teleost granulocyte colony-stimulating factor receptor

The granulocyte colony stimulating factor receptor (*gcsfr*) has been identified in zebrafish [412], goldfish [423], and trout (AJ616901). Only one gene copy has been identified, although Southern blotting for goldfish *gcsfr* suggested the presence of more than one gene [423]. Analysis of the upstream promoter region of the 16 exon zebrafish *gcsfr* gene showed conserved putative sites for binding of the transcription factors HOXA5, PU.1 and CEBP family members [412], similar to the human *gcsfr* promoter region. These data suggest the conserved regulation of *gcsfr* gene expression in teleosts.

The predicted protein structure of zebrafish and goldfish GCSFRs is conserved across vertebrates. The teleost GCSFR extracellular domain is comprised of a signal peptide, an Ig-like domain, a cytokine homology domain containing the WSXWS motif and four cysteine residues, and three fibronectin domains. Following the transmembrane region, the intracellular region contains predicted Box1, Box2, and Box 3 signaling motifs and 6 tyrosine residues [412, 423], shown to be involved in receptor activation and internalization in higher vertebrates.

In zebrafish, the *gcsfr* mRNA is expressed as early as 14 hpf in the RBI, followed by the yolk sac, the ICM, and finally in the kidney by 96 hpf, consistent with the production of neutrophils during primitive and definitive hematopoiesis. In adult goldfish, *gcsfr* mRNA levels were highest in kidney and spleen, followed by the gill, intestine, heart, brain and blood [423]. The *gcsfr* mRNA was highly expressed in goldfish neutrophils, and was up-regulated in response to mitogens or pathogens [423] (see chapter 8 of this thesis),

2.4.3 Transcription factors

In addition to the transcription factors described in section 1.3.3 of this thesis, there are a number of transcription factors downstream that participate in determining GMP fate decisions and that play a role in macrophage and neutrophil cell development, reviewed by [41, 444].

2.4.3.1 Early growth response (Egr)

The four Egr proteins, EGR1 [445, 446], EGR2 [447], EGR3 [448] and EGR4 [449], are members of the zinc finger transcription factor family and have an N-terminus activation domain, a repressor domain capable of binding to NAB1/2, and a DNA binding domain comprised of three zinc fingers that bind to the GC rich sequence, 5'-GCGGGGGC'3' [450]. EGR1 promotes commitment to the macrophage lineage at the expense of granulocytic lineage [451, 452] and has been shown to be essential for myeloblast differentiation into monocytes/macrophages [453, 454]. Treatment of mouse bone marrow cells with CSF-1 has been shown to induce *EGR1* mRNA levels by 6-7 fold three hours post treatment, as well as *EGR2* and *EGR3* mRNA levels by 2-4 fold [455]. In addition, GM-CSF, IL-3 and GCSF can also increase *EGR1*, *EGR2*, and *EGR3* mRNA levels in bone marrow cells [455], although there is conflicting evidence for the response of *EGR1* expression to GCSF treatment [451]. Although $EGR^{-/-}$ mice display normal macrophage development [456], it is thought that there is redundancy amongst the Egr transcription factors. Consistent with this idea, EGR2 is also abundant in monoblasts and monocytes [457], and may be involved

in monocyte differentiation. Although a zebrafish orthologue of *EGR1* has been identified [458], the role of *egr1* in teleost macrophage development has not been examined.

2.4.3.2 Growth factor independence 1 (Gfi1)

Growth factor independence 1 (GFI1) is a zinc finger transcription factor comprised of an N-terminal Snail/Gfi1 (SNAG) domain that is involved in recruiting proteins to modify histones, and a C-terminal domain containing six zinc fingers involved in DNA recognition [459]. *GFI1* is expressed in T-cells, B-cells, mature granulocytes and activated macrophages [460, 461]. GFI1$^{-/-}$ mice showed slight defects in lymphocyte development, increased monocyte and monocyte precursor numbers, and an absence of granulocytes [460, 461]. These knockout mice were highly susceptible to infections and only survived for 1-2 months [460, 461]. Furthermore, myeloid progenitors from GFI1$^{-/-}$ mice did not differentiate into mature granulocytes in the presence of GCSF *in vitro* [460] or *in vivo* [461]. C/EBPα can up-regulate *GFI1* expression, promoting a neutrophil cell fate, and GFI1 also acts as a negative regulator on *PU.1* to decrease its expression [287, 290], and this lower level of *PU.1* drives granulocyte cell fate [285, 287]. GFI1 is important for neutrophil differentiation [287, 460, 462] and acts by activating Ras guanine nucleotide releasing protein 1 (RasGRP1) which is necessary for activating Ras in the Ras/MEK/Erk pathway that is initiated during GCSF signaling [463]. The expression of GFI1 is sustained during differentiation and the transcription factor functions by blocking the expression of *EGR-2/NAB-2*, effectively antagonizing the EGR1/2 transcription factor and

preventing initiation of a monocytic differentiation pathway, thereby promoting neutrophil differentiation [287, 462]. *GFI1* has a binding site for EGR-1-2/NAB-2 in its upstream promoter region, leading to repression of *GFI1* expression and blocking neutrophil differentiation [287]. Conversely, several binding sites for GFI1 were identified in the promoter regions of *EGR-1* and *EGR-2*, and GFI1 was shown to repress transcription of the Egr genes [287]. These experiments demonstrate that, like PU.1 and GATA1, GFI1 and EGR-1/EGR-2 act as an antagonistic pair to regulate neutrophil versus macrophage lineage fate.

In zebrafish, two *gfi1* genes have been identified, termed *gfi1* and *gfi1.1*. *gfi1* is primarily expressed in neural tissues, and not in the hematopoietic system [464], suggesting that this is not the functional orthologue of mammalian GFI1. However, *gfi1.1* was expressed in the different hematopoietic organs of the developing zebrafish embryo, suggesting that *gfi1.1* is expressed in hematopoietic cells [465]. Zebrafish *gfi1.1* morphants displayed a three-fold increase in the number of *pu.1*$^{+}$ cells, along with an increase in *l-plastin* expression and a decrease in *mpo* expression [465]. These data are consistent with the known functional role of mammalian GFI1, suggesting that zebrafish GFI1.1, and not zebrafish GFI1, is the functional orthologue of mammalian GFI.

2.4.3.3 Interferon response factor-8 (IRF-8)

Interferon response factor-8 (IRF-8, also known as ICSBP) is one out of nine members of the IRF transcription factor family and is characterized by an N-terminal DNA binding domain and a C-terminus IRF association domain that can associate with other IRF or Ets transcription family members, reviewed by [466,

467]. IRF8$^{-/-}$ mice and BXH-2 mice with a mutation in their IRF association domain show a drastic expansion of granulocytes at the expense of macrophages [468, 469]. Enforced expression of *IRF8* in myeloid progenitor cells *in vitro* led to the induced expression of a number of macrophage lineage differentiation transcripts including *CSF-1R* and *EGR1*. Additionally, enforced expression of *IRF8* in myeloid cell lines prevented their differentiation into granulocytes when treated with GCSF [470]. Furthermore, interferon gamma produced during infection acted on GMPs to up-regulate *IRF8* and *PU.1* expression which promoted monocyte development at the expense of neutrophil development in the presence of CSF-1 [471]. Interferon gamma was also found to inhibit the proliferation and differentiation of CMP and GMPs in the presence of GCSF by up-regulating the expression of suppressor of cytokine signaling 3 (SOCS3) to negatively feedback on the GCSFR signaling pathway [471]. It is clear from the *in vivo* and *in vitro* studies that IRF8 promotes the commitment of myeloid progenitors along the macrophage lineage at the expense of the granulocyte lineage.

The homologue of *irf-8* has been identified in rainbow trout [472] and zebrafish [473] with 53-55% amino acid identity to human IRF8 [472, 473]. In trout tissues, *irf8* mRNA was detected in the spleen, head kidney, gill, brain, intestine, skin, muscle, and liver [472] and mRNA levels could be up-regulated in splenocytes upon treatment with Poly I:C, PMA, PHA and recombinant IL-15. However, the role of IRF8 in GMP fate decisions or during macrophage development was not assessed. In zebrafish developing embryos, *irf8* mRNA was first detected in the rostral blood island, the site of primitive myelopoiesis, and was found to be co-expressed with *csf-1r* mRNA, but not in cells positive for

mpx, suggesting that *irf8* is expressed in cells committed to the macrophage lineage [473]. In zebrafish *irf8* morphants, *csf-1r*$^{+}$ cells were absent, while *mpx*$^{+}$ cells and mature neutrophils were increased by approximately three-fold, suggesting IRF8 is required for macrophage development. This phenotype could be rescued by injecting embryos with *irf8* mRNA. Conversely, the over-expression of *irf8* mRNA in zebrafish resulted in an increase in macrophages by approximately 50% and a decrease in neutrophil numbers by about 40% [473]. These data are similar to those of the mammalian system and suggest a conserved role for IRF8 in determining macrophage over neutrophil cell lineage during primitive myelopoiesis. However, whether IRF8 plays the same role during definitive myelopoiesis in teleosts remains to be determined.

2.4.3.4 MafB

In addition to the previously described role of MAFB in HSCs and CMPs (see section 1.3.3.1), *MAFB* is highly expressed in monocytes and macrophages [274, 474] and has been shown to induce differentiation of myeloblasts into monocytes and macrophages [2, 457, 475, 476]. Furthermore, MAFB and c-MAF double knockout mice displayed differentiated macrophages that were capable of proliferating in response to CSF-1 in semi-solid and liquid culture [476]. Therefore, it appears that *MAFB* expression is sustained in monocytes and macrophages in order to prevent proliferation in these terminally differentiated cell populations.

Although studies examining the role of MAFB in teleost myelopoiesis are limited, studies in the goldfish system identified a *mafb* transcript and showed

that *mafb* mRNA levels increased with macrophage development in the goldfish primary kidney macrophage culture system [294], comprising of progenitors, monocytes and macrophages. The increasing mRNA levels of *mafb* during macrophage differentiation are similar to what has been observed in mammalian systems and suggest that MAFB may play a role in teleost macrophage differentiation.

2.5 Goldfish model system

Studies in the Belosevic laboratory have developed a unique *in vitro* derived goldfish primary kidney macrophage (PKM) culture system as a model system for the *in vitro* study of teleost monopoiesis. Initial experiments demonstrated the capacity of small mononuclear cells isolated from the kidney to proliferate *in vitro* when seeded above a certain cell density, or at a lower cell density when supplemented with cell-conditioned medium (CCM) from previous cultures [477, 478]. Furthermore, these kidney leukocytes were capable of differentiation over 8-10 days of cultivation and flow cytometry analysis of leukocytes from the PKM cultures, based on forward and side scatter, revealed the presence of three cell sub-populations, R1-, R2- and R3-gated cells (Fig. 2.1) [477, 478]. The spontaneous proliferation and differentiation of kidney leukocytes suggested the production of endogenous growth factors by these cells [477, 478].

The R1, R2 and R3 cell subpopulations were characterized based on cytochemistry and functional assays. R1 cells had low forward and side scatter, indicative of small size (6-10 μm in diameter) and low internal complexity. They had a high nucleus to cytoplasm ratio, were positive for acid phosphatase, and

negative for myeloperoxidase and non-specific esterase [478]. The R2 cells had a higher forward scatter, indicative of a larger cell size (12-20 μm in diameter), and low side scatter representing a less internally complex cell [478]. R2 cells were positive for acid phosphatase, variable for non-specific esterase, and showed localized staining for myeloperoxidase [478]. Functional characterization of the R2 gated PKMs demonstrated their capacity to mount antimicrobial functions such as phagocytosis of bacteria and the production of toxic reactive oxygen and nitrogen intermediates [477, 478]. The R3 cells were intermediate in size (12-15 μm in diameter) and internally complex [478]. The R3 cells were acid-phosphatase positive, non-specific esterase variable, and showed diffuse staining for myeloperoxidase [478]. Functionally, the R3 cells were capable of producing reactive oxygen intermediates, but did not produce significant amounts of nitric oxide [478]. The characterization of these cell sub-populations demonstrated the presence of putative progenitor cells (R1 gate), monocytes (R3 gate), and mature macrophages (R2 gate) in PKM cultures and represent distinct junctures of macrophage development (Fig. 2.1) [477, 478].

The production of endogenous growth factors by leukocytes within the PKM cultures prompted the examination of the target cell sub-population(s) upon which they acted and their effects on cell proliferation and differentiation. Sorted R1 cells proliferated and differentiated into macrophages in the absence of CCM, and the proliferation and differentiation could be enhanced in the presence of CCM [478]. These data suggest that R1 cells may be a source of endogenous growth factors that act in an autocrine and paracrine manner. R2 cells proliferated only in the presence of CCM, and appeared to be terminally differentiated [478]. The proliferation of R2 cells demonstrated goldfish

macrophages are capable of self-renewal [478, 479]. Monocytes sorted from the R3 gate differentiated into macrophages when treated with CCM, but failed to proliferate [478]. Clearly, different endogenous growth factors present in CCM exert distinct actions on macrophage cell sub-populations.

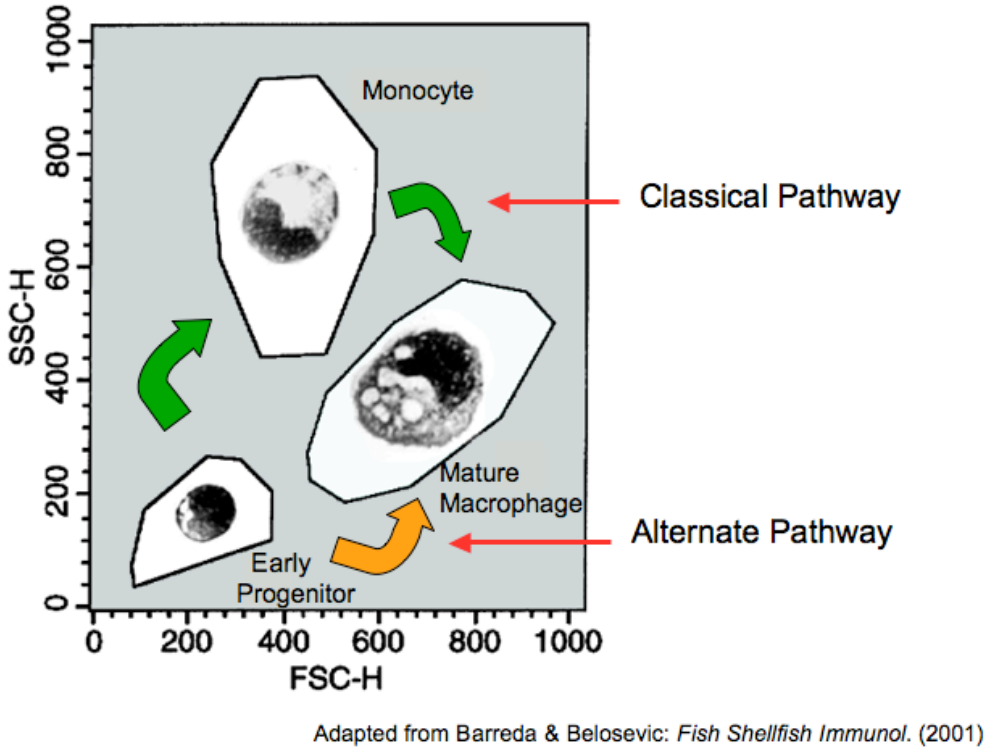
Two pathways of macrophage development were proposed to occur in the PKM cultures. The predominant pathway was classical macrophage development in which progenitor cells differentiated into monocytes and then macrophages [479]. The second was an alternative pathway of macrophage development in which progenitor cells differentiated into macrophages without a prominent monocytic stage [479] and was likened to the production of primitive macrophages during embryonic development. The possible retention of the alternative pathway of macrophage production in addition to the classical pathway may provide a mechanism for rapid generation of macrophages during injury or infection *in vivo*.

The observed kinetics of the PKM cultures suggested three phases of growth. Initially, there is a lag phase (days 1-4) where many cells die, followed by a proliferative phase (days 5-9) where cell numbers rapidly increase [477], and finally, a senescence phase (days 10-14) characterized by cell clumping and cell apoptosis [479, 480]. Differential cross screening of proliferative versus senescence phase PKMs identified a number of differentially expressed genes including those involved in hematopoiesis, signal transduction, transcription, translation and protein processing [294]. The involvement of the identified transcripts in the regulation of cell development [85, 382, 481] will be discussed in the following sections.

These seminal observations from PKM cultures established three important ideas regarding goldfish monopoiesis: (1) kidney leukocytes produce their own endogenous growth factors important for driving proliferation and differentiation [477, 478]. (2) Within the population of small leukocyte R1 cells, a population of macrophage progenitor cells must exist. (3) Unlike mammalian systems, the progenitor cell population gives rise to fully differentiated macrophages *in vitro* in the absense of exogenous growth factors. Thus, the goldfish PKM model system allows for comprehensive analysis of the interactions between developing macrophage subpopulations *in vitro*.

2.6 Summary

Myelopoiesis is an orchestration of a multitude of growth factors and transcription factors that control cell fate decisions and differentiation along a chosen cell lineage. It is evident that there exists some functional redundancy in the action of myelopoietic growth factors, most likely put in place to ensure the necessary production of these critical innate immune cells. Studies have focused on examining the regulation of myelopoiesis in the mouse model system, and have only just begun in the teleost model system. The loss of key myeloid growth factors (i.e. IL-3 and GM-CSF) and the addition of new genes due to whole genome duplication events with the potential for sub-functionalization or neo-functionalization suggests that although myelopoiesis is a conserved process amongst vertebrates, the growth factors and transcription factors controlling cell fate decisions and differentiation may differ between mammals and teleosts.



Adapted from Barreda & Belosevic: *Fish Shellfish Immunol.* (2001)

Figure 2.1: Pathways of goldfish macrophage development.
Early progenitor cells fall within the R1 gate, monocytes fall within the R3 gate and mature macrophages fall within the R2 gate.

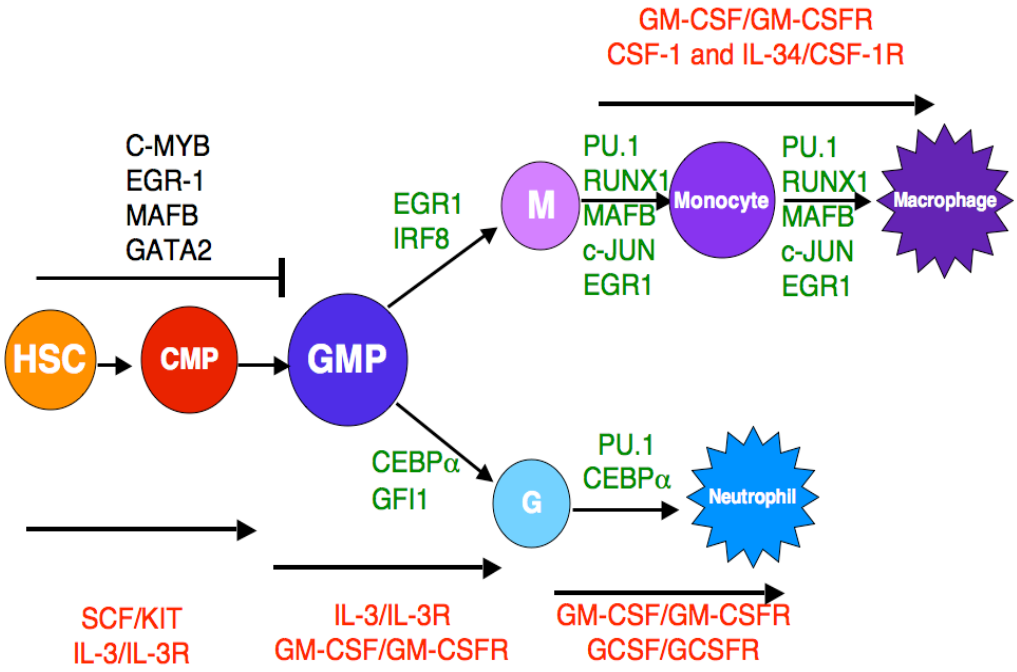


Figure 2.2: Summary of myeloid cell development and the important growth factors, receptors, and transcription factors.
Transcription factors that positively regulate myeloid cell development and promote lineage commitment are shown in green, while transcription factors that are involved in retaining early hematopoietic cell phenotype are shown in black. Growth factors and their receptors are shown in red, and the arrow denotes the cell types they act upon. Figure key: (1) **Cellular stages:** HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) **Transcription factors:** C-MYB, cellular myelobastosis oncogene; EGR-1, early growth response-1; MAFB, musculoaponeurotic fibrosarcoma oncogene homologue B; GATA2, GATA binding protein 2; IRF8, interferon regulatory factor 8; CEBPα, CCAAT/enhancer-binding protein alpha; GFI1, growth factor independent 1; RUNX1, runt-related transcription factor 1. (3) **Growth factors:** SCF, stem cell factor; IL-3, interleukin-3; GM-CSF, granulocyte-macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1 (macrophage colony-stimulating factor); GCSF, granulocyte colony-stimulating factor. (4) **Receptors:** IL-3R, interleukin 3 receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; CSF-1R, colony-stimulating factor-1 receptor (macrophage colony-stimulating factor receptor); GCSFR, granulocyte colony-stimulating factor receptor.

2.7 References

[1] **Martinez-Agosto JA, Mikkola HK, Hartenstein V, Banerjee U.** The hematopoietic stem cell and its niche: a comparative view. Genes Dev. 2007;21(23):3044-60.

[2] **Sarrazin S, Mossadegh-Keller N, Fukao T, Aziz A, Mourcin F, Vanhille L, et al.** MafB restricts M-CSF-dependent myeloid commitment divisions of hematopoietic stem cells. Cell. 2009;138(2):300-13.

[3] **Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC, Schroeder T.** Hematopoietic cytokines can instruct lineage choice. Science. 2009;325(5937):217-8.

[4] **Sarrazin S, Sieweke M.** Integration of cytokine and transcription factor signals in hematopoietic stem cell commitment. Semin Immunol. 2011;23(5):326-34.

[5] **Dzierzak E.** The emergence of definitive hematopoietic stem cells in the mammal. Curr Opin Hematol. 2005;12(3):197-202.

[6] **Baron MH, Fraser ST.** The specification of early hematopoiesis in the mammal. Curr Opin Hematol. 2005;12(3):217-21.

[7] **Wang LD, Wagers AJ.** Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol. 2011;12(10):643-55.

[8] **McCulloch EA, Till JE.** The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res. 1960;13115-25.

[9] **Till JE, Mc CE.** A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14213-22.

[10] **McCulloch EA, Till JE.** The sensitivity of cells from normal mouse bone marrow to gamma radiation *in vitro* and *in vivo*. Radiat Res. 1962;16822-32.

[11] **Becker AJ, Mc CE, Till JE.** Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963;197452-4.

[12] **Medvinsky AL, Dzierzak EA.** Development of the definitive hematopoietic hierarchy in the mouse. Dev Comp Immunol. 1998;22(3):289-301.

[13] **Herbomel P, Thisse B, Thisse C.** Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development. 1999;126(17):3735-45.

[14] **Le Guyader D, Redd MJ, Colucci-Guyon E, Murayama E, Kissa K, Briolat V, et al.** Origins and unconventional behavior of neutrophils in developing zebrafish. Blood. 2008;111(1):132-41.

[15] **Detrich HW, 3rd, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, et al.** Intraembryonic hematopoietic cell migration during vertebrate development. Proc Natl Acad Sci U S A. 1995;92(23):10713-7.

[16] **Jin H, Xu J, Wen Z.** Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. Blood. 2007;109(12):5208-14.

[17] **Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D.** Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. Development. 2007;134(23):4147-56.

[18] **Davidson AJ, Zon LI.** The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. Oncogene. 2004;23(43):7233-46.

[19] **Chen AT, Zon LI.** Zebrafish blood stem cells. J Cell Biochem. 2009;108(1):35-42.

[20] **de Jong JL, Zon LI.** Use of the zebrafish system to study primitive and definitive hematopoiesis. Annu Rev Genet. 2005;39481-501.

[21] **Zapata A, Diez B, Cejalvo T, Gutierrez-de Frias C, Cortes A.** Ontogeny of the immune system of fish. Fish Shellfish Immunol. 2006;20(2):126-36.

[22] **Rombout JH, Huttenhuis HB, Picchietti S, Scapigliati G.** Phylogeny and ontogeny of fish leucocytes. Fish Shellfish Immunol. 2005;19(5):441-55.

[23] **Bertrand JY, Kim AD, Teng S, Traver D.** $CD41^{+}$ $cmyb^{+}$ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. Development. 2008;135(10):1853-62.

[24] **Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, et al.** Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity. 2006;25(6):963-75.

[25] **Willett CE, Cortes A, Zuasti A, Zapata AG.** Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn. 1999;214(4):323-36.

[26] **Zapata A.** Ultrastructural study of the teleost fish kidney. Dev Comp Immunol. 1979;3(1):55-65.

[27] **Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI.** Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. Nat Immunol. 2003;4(12):1238-46.

[28] **Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, et al.** Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004;104(5):1298-305.

[29] **Nakanishi T, Ototake M.** The graft-versus-host reaction (GVHR) in the ginbuna crucian carp, *Carassius auratus langsdorfii.* Dev Comp Immunol. 1999;23(1):15-26.

[30] **Moritomo T, Asakura N, Sekiya M, Ototake M, Inoue Y, Nakanishi T.** Cell culture of clonal ginbuna crucian carp hematopoietic cells: differentiation of cultured cells into erythrocytes *in vivo*. Dev Comp Immunol. 2004;28(9):863-9.

[31] **Kobayashi I, Sekiya M, Moritomo T, Ototake M, Nakanishi T.** Demonstration of hematopoietic stem cells in ginbuna carp (*Carassius auratus langsdorfii*) kidney. Dev Comp Immunol. 2006;30(11):1034-46.

[32] **Kobayashi I, Saito K, Moritomo T, Araki K, Takizawa F, Nakanishi T.** Characterization and localization of side population (SP) cells in zebrafish kidney hematopoietic tissue. Blood. 2008;111(3):1131-7.

[33] **Kobayashi I, Moritomo T, Ototake M, Nakanishi T.** Isolation of side population cells from ginbuna carp (*Carassius auratus langsdorfii*) kidney hematopoietic tissues. Dev Comp Immunol. 2007;31(7):696-707.

[34] **Kobayashi I, Kuniyoshi S, Saito K, Moritomo T, Takahashi T, Nakanishi T.** Long-term hematopoietic reconstitution by transplantation of kidney hematopoietic stem cells in lethally irradiated clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Dev Comp Immunol. 2008;32(8):957-65.

[35] **Kobayashi I, Kusakabe H, Toda H, Moritomo T, Takahashi T, Nakanishi T.** *In vivo* characterization of primitive hematopoietic cells in clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Vet Immunol Immunopathol. 2008;126(1-2):74-82.

[36] **Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE.** Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood. 2001;98(10):3087-96.

[37] **Metcalf D.** Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation. Blood. 1998;92(2):345-7; discussion 52.

[38] **Metcalf D.** Hematopoietic cytokines. Blood. 2008;111(2):485-91.

[39] **Zhu J, Emerson SG.** Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene. 2002;21(21):3295-313.

[40] **Cantor AB, Orkin SH.** Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene. 2002;21(21):3368-76.

[41] **Friedman AD.** Transcriptional regulation of granulocyte and monocyte development. Oncogene. 2002;21(21):3377-90.

[42] **Ye M, Graf T.** Early decisions in lymphoid development. Curr Opin Immunol. 2007;19(2):123-8.

[43] **Lai AY, Kondo M.** Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. J Exp Med. 2006;203(8):1867-73.

[44] **Adolfsson J, Mansson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, et al.** Identification of $Flt3^+$ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell. 2005;121(2):295-306.

[45] **Chi AW, Chavez A, Xu L, Weber BN, Shestova O, Schaffer A, et al.** Identification of Flt3CD150 myeloid progenitors in adult mouse bone marrow that harbor T lymphoid developmental potential. Blood. 2011;118(10):2723-32.

[46] **Ng SY, Yoshida T, Zhang J, Georgopoulos K.** Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. Immunity. 2009;30(4):493-507.

[47] **Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, et al.** Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell. 2007;1(4):428-42.

[48] **Montecino-Rodriguez E, Leathers H, Dorshkind K.** Bipotential B-macrophage progenitors are present in adult bone marrow. Nat Immunol. 2001;2(1):83-8.

[49] **Kondo M, Weissman IL, Akashi K.** Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91(5):661-72.

[50] **Luc S, Buza-Vidas N, Jacobsen SE.** Biological and molecular evidence for existence of lymphoid-primed multipotent progenitors. Ann N Y Acad Sci. 2007;110689-94.

[51] **Zijlmans JM, Visser JW, Kleiverda K, Kluin PM, Willemze R, Fibbe WE.** Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. Proc Natl Acad Sci U S A. 1995;92(19):8901-5.

[52] **Spangrude GJ, Johnson GR.** Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci U S A. 1990;87(19):7433-7.

[53] **Wolf NS, Kone A, Priestley GV, Bartelmez SH.** *In vivo* and *in vitro* characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp Hematol. 1993;21(5):614-22.

[54] **Gallacher L, Murdoch B, Wu DM, Karanu FN, Keeney M, Bhatia M.** Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7. Blood. 2000;95(9):2813-20.

[55] **Uchida N, Weissman IL.** Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med. 1992;175(1):175-84.

[56] **Morrison SJ, Weissman IL.** The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity. 1994;1(8):661-73.

[57] **Osawa M, Nakamura K, Nishi N, Takahasi N, Tokuomoto Y, Inoue H, et al.** In vivo self-renewal of c-Kit^{+} Sca-1^{+} Lin(low/-) hemopoietic stem cells. J Immunol. 1996;156(9):3207-14.

[58] **Wagers AJ, Weissman IL.** Differential expression of alpha2 integrin separates long-term and short-term reconstituting Lin-/loThy1.1(lo)c-kit^{+} Sca-1^{+} hematopoietic stem cells. Stem Cells. 2006;24(4):1087-94.

[59] **Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Miura Y, Suda T.** *In vivo* and *in vitro* stem cell function of c-kit^{-} and Sca-1^{-} positive murine hematopoietic cells. Blood. 1992;80(12):3044-50.

[60] **Simmons DL, Satterthwaite AB, Tenen DG, Seed B.** Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. J Immunol. 1992;148(1):267-71.

[61] **Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH.** Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol. 1984;133(1):157-65.

[62] **Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, et al.** Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. J Clin Invest. 1988;81(3):951-5.

[63] **Katz FE, Tindle R, Sutherland DR, Greaves MF.** Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. Leuk Res. 1985;9(2):191-8.

[64] **Andrews RG, Singer JW, Bernstein ID.** Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood. 1986;67(3):842-5.

[65] **Molgaard HV, Spurr NK, Greaves MF.** The hemopoietic stem cell antigen, CD34, is encoded by a gene located on chromosome 1. Leukemia. 1989;3(11):773-6.

[66] **Izawa K, Tani K, Nakazaki Y, Hibino H, Sugiyama H, Kawasaki A, et al.** Hematopoietic activity of common marmoset CD34 cells isolated by a novel monoclonal antibody MA24. Exp Hematol. 2004;32(9):843-51.

[67] **Brown J, Greaves MF, Molgaard HV.** The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain, and embryonic fibroblasts. Int Immunol. 1991;3(2):175-84.

[68] **McSweeney PA, Rouleau KA, Storb R, Bolles L, Wallace PM, Beauchamp M, et al.** Canine CD34: cloning of the cDNA and evaluation of an antiserum to recombinant protein. Blood. 1996;88(6):1992-2003.

[69] **Niku M, Pessa-Morikawa T, Ra R, Ekman A, Iivanainen A.** Expression of CD34 mRNA and protein in cattle. Vet Immunol Immunopathol. 2007;117(3-4):162-72.

[70] **Zhou JH, Hikono H, Ohta M, Sakurai M.** Cloning of bovine CD34 cDNA. J Vet Med Sci. 2001;63(9):1051-3.

[71] **Tenen DG, Satterthwaite AB, Borson R, Simmons D, Eddy RL, Shows TB.** Chromosome 1 localization of the gene for CD34, a surface antigen of human stem cells. Cytogenet Cell Genet. 1990;53(1):55-7.

[72] **Howell DG, Molgaard HV, Greaves MF, Spurr NK.** Localization of the gene coding for the hemopoietic stem cell antigen $CD34^+$ to chromosome 1q32. Human Genet. 1991;87625-27.

[73] **Satterthwaite AB, Burn TC, Le Beau MM, Tenen DG.** Structure of the gene encoding CD34, a human hematopoietic stem cell antigen. Genomics. 1992;12(4):788-94.

[74] **Suda J, Sudo T, Ito M, Ohno N, Yamaguchi Y, Suda T.** Two types of murine CD34 mRNA generated by alternative splicing. Blood. 1992;79(9):2288-95.

[75] **Nakamura Y, Komano H, Nakauchi H.** Two alternative forms of cDNA encoding CD34. Exp Hematol. 1993;21(2):236-42.

[76] **Sassetti C, Van Zante A, Rosen SD.** Identification of endoglycan, a member of the CD34/podocalyxin family of sialomucins. J Biol Chem. 2000;275(12):9001-10.

[77] **McNagny KM, Pettersson I, Rossi F, Flamme I, Shevchenko A, Mann M, et al.** Thrombomucin, a novel cell surface protein that defines thrombocytes and multipotent hematopoietic progenitors. J Cell Biol. 1997;138(6):1395-407.

[78] **Furness SG, McNagny K.** Beyond mere markers: functions for CD34 family of sialomucins in hematopoiesis. Immunol Res. 2006;34(1):13-32.

[79] **Holyoake TL, Alcorn MJ.** $CD34^+$ positive haemopoietic cells: biology and clinical applications. Blood Rev. 1994;8(2):113-24.

[80] **Krause DS, Fackler MJ, Civin CI, May WS.** CD34: structure, biology, and clinical utility. Blood. 1996;87(1):1-13.

[81] **Engelhardt M, Lubbert M, Guo Y.** CD34(+) or CD34(-): which is the more primitive? Leukemia. 2002;16(9):1603-8.

[82] **Fargeas CA, Florek M, Huttner WB, Corbeil D.** Characterization of prominin-2, a new member of the prominin family of pentaspan membrane glycoproteins. J Biol Chem. 2003;278(10):8586-96.

[83] **Husain SM, Shou Y, Sorrentino BP, Handgretinger R.** Isolation, molecular cloning and in vitro expression of rhesus monkey (Macaca mulatta) prominin-1.s1 complementary DNA encoding a potential hematopoietic stem cell antigen. Tissue Antigens. 2006;68(4):317-24.

[84] **Han Z, Papermaster DS.** Identification of three prominin homologs and characterization of their messenger RNA expression in Xenopus laevis tissues. Mol Vis. 2011;171381-96.

[85] **Walsh JG, Barreda DR, Belosevic M.** Cloning and expression analysis of goldfish (*Carassius auratus* L.) prominin. Fish Shellfish Immunol. 2007;22(4):308-17.

[86] **McGrail M, Batz L, Noack K, Pandey S, Huang Y, Gu X, et al.** Expression of the zebrafish CD133/prominin1 genes in cellular proliferation zones in the embryonic central nervous system and sensory organs. Dev Dyn. 2010;239(6):1849-57.

[87] **Weigmann A, Corbeil D, Hellwig A, Huttner WB.** Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci U S A. 1997;94(23):12425-30.

[88] **Corbeil D, Roper K, Weigmann A, Huttner WB.** AC133 hematopoietic stem cell antigen: human homologue of mouse kidney prominin or distinct member of a novel protein family? Blood. 1998;91(7):2625-6.

[89] **Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al.** A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood. 1997;90(12):5013-21.

[90] **Fargeas CA, Corbeil D, Huttner WB.** AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in need of a rational nomenclature. Stem Cells. 2003;21(4):506-8.

[91] **Fargeas CA, Huttner WB, Corbeil D.** Nomenclature of prominin-1 (CD133) splice variants - an update. Tissue Antigens. 2007;69(6):602-6.

[92] **Fargeas CA, Joester A, Missol-Kolka E, Hellwig A, Huttner WB, Corbeil D.** Identification of novel Prominin-1/CD133 splice variants with alternative C-termini and their expression in epididymis and testis. J Cell Sci. 2004;117(Pt 18):4301-11.

[93] **Kemper K, Tol MJ, Medema JP.** Mouse tissues express multiple splice variants of prominin-1. PLoS One. 2010;5(8):e12325.

[94] **Corbeil D, Joester A, Fargeas CA, Jaszai J, Garwood J, Hellwig A, et al.** Expression of distinct splice variants of the stem cell marker prominin-1 (CD133) in glial cells. Glia. 2009;57(8):860-74.

[95] **Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al.** AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90(12):5002-12.

[96] **Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, et al.** The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem. 2000;275(8):5512-20.

[97] **Kania G, Corbeil D, Fuchs J, Tarasov KV, Blyszczuk P, Huttner WB, et al.** Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors. Stem Cells. 2005;23(6):791-804.

[98] **Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ.** Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res. 2005;65(23):10946-51.

[99] **Vander Griend DJ, Karthaus WL, Dalrymple S, Meeker A, DeMarzo AM, Isaacs JT.** The role of CD133 in normal human prostate stem cells and malignant cancer-initiating cells. Cancer Res. 2008;68(23):9703-11.

[100] **Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al.** Identification of human brain tumour initiating cells. Nature. 2004;432(7015):396-401.

[101] **Chearwae W, Bright JJ.** PPARgamma agonists inhibit growth and expansion of $CD133^{+}$ brain tumour stem cells. Br J Cancer. 2008;99(12):2044-53.

[102] **Neuzil J, Stantic M, Zobalova R, Chladova J, Wang X, Prochazka L, et al.** Tumour-initiating cells vs. cancer 'stem' cells and CD133: what's in the name? Biochem Biophys Res Commun. 2007;355(4):855-9.

[103] **Mizrak D, Brittan M, Alison MR.** CD133: molecule of the moment. J Pathol. 2008;214(1):3-9.

[104] **Bidlingmaier S, Zhu X, Liu B.** The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. J Mol Med (Berl). 2008;86(9):1025-32.

[105] **Keysar SB, Jimeno A.** More than markers: biological significance of cancer stem cell-defining molecules. Mol Cancer Ther. 2010;9(9):2450-7.

[106] **Roper K, Corbeil D, Huttner WB.** Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. Nat Cell Biol. 2000;2(9):582-92.

[107] **Florek M, Bauer N, Janich P, Wilsch-Braeuninger M, Fargeas CA, Marzesco AM, et al.** Prominin-2 is a cholesterol-binding protein associated with apical and basolateral plasmalemmal protrusions in polarized epithelial cells and released into urine. Cell Tissue Res. 2007;328(1):31-47.

[108] **Corbeil D, Roper K, Hannah MJ, Hellwig A, Huttner WB.** Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells - a combination of apical sorting and retention in plasma membrane protrusions. J Cell Sci. 1999;112 (Pt 7)1023-33.

[109] **Maw MA, Corbeil D, Koch J, Hellwig A, Wilson-Wheeler JC, Bridges RJ, et al.** A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. Hum Mol Genet. 2000;9(1):27-34.

[110] **Taieb N, Maresca M, Guo XJ, Garmy N, Fantini J, Yahi N.** The first extracellular domain of the tumour stem cell marker CD133 contains an antigenic ganglioside-binding motif. Cancer Lett. 2009;278(2):164-73.

[111] **Yang Z, Chen Y, Lillo C, Chien J, Yu Z, Michaelides M, et al.** Mutant prominin 1 found in patients with macular degeneration disrupts photoreceptor disk morphogenesis in mice. J Clin Invest. 2008;118(8):2908-16.

[112] **Boivin D, Labbe D, Fontaine N, Lamy S, Beaulieu E, Gingras D, et al.** The stem cell marker CD133 (prominin-1) is phosphorylated on cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. Biochemistry. 2009;48(18):3998-4007.

[113] **Gillette JM, Larochelle A, Dunbar CE, Lippincott-Schwartz J.** Intercellular transfer to signalling endosomes regulates an ex vivo bone marrow niche. Nat Cell Biol. 2009;11(3):303-11.

[114] **Bauer N, Wilsch-Brauninger M, Karbanova J, Fonseca AV, Strauss D, Freund D, et al.** Haematopoietic stem cell differentiation promotes the release of prominin-1/CD133-containing membrane vesicles--a role of the endocytic-exocytic pathway. EMBO Mol Med. 2011;3(7):398-409.

[115] **Jaszai J, Fargeas CA, Graupner S, Tanaka EM, Brand M, Huttner WB, et al.** Distinct and conserved prominin-1/CD133-positive retinal cell populations identified across species. PLoS One. 2011;6(3):e17590.

[116] **Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, et al.** The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell. 1990;63(1):225-33.

[117] **Copeland NG, Gilbert DJ, Cho BC, Donovan PJ, Jenkins NA, Cosman D, et al.** Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell. 1990;63(1):175-83.

[118] **Witte ON.** Steel locus defines new multipotent growth factor. Cell. 1990;63(1):5-6.

[119] **Zhang Z, Zhang R, Joachimiak A, Schlessinger J, Kong XP.** Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. Proc Natl Acad Sci U S A. 2000;97(14):7732-7.

[120] **Russell ES.** Hereditary anemias of the mouse: a review for geneticists. Adv Genet. 1979;20357-459.

[121] **Dexter TM, Moore MA.** In vitro duplication and "cure" of haemopoietic defects in genetically anaemic mice. Nature. 1977;269(5627):412-4.

[122] **Anderson DM, Williams DE, Tushinski R, Gimpel S, Eisenman J, Cannizzaro LA, et al.** Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. Cell Growth Differ. 1991;2(8):373-8.

[123] **Geissler EN, Liao M, Brook JD, Martin FH, Zsebo KM, Housman DE, et al.** Stem cell factor (SCF), a novel hematopoietic growth factor and ligand for c-kit tyrosine kinase receptor, maps on human chromosome 12 between 12q14.3 and 12qter. Somat Cell Mol Genet. 1991;17(2):207-14.

[124] **Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, et al.** Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell. 1990;63(1):235-43.

[125] **Langley KE, Bennett LG, Wypych J, Yancik SA, Liu XD, Westcott KR, et al.** Soluble stem cell factor in human serum. Blood. 1993;81(3):656-60.

[126] **Arakawa T, Yphantis DA, Lary JW, Narhi LO, Lu HS, Prestrelski SJ, et al.** Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. J Biol Chem. 1991;266(28):18942-8.

[127] **Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, et al.** Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell. 2002;109(5):625-37.

[128] **Kridel SJ, Chen E, Kotra LP, Howard EW, Mobashery S, Smith JW.** Substrate hydrolysis by matrix metalloproteinase-9. J Biol Chem. 2001;276(23):20572-8.

[129] **Pandiella A, Bosenberg MW, Huang EJ, Besmer P, Massague J.** Cleavage of membrane-anchored growth factors involves distinct protease activities regulated through common mechanisms. J Biol Chem. 1992;267(33):24028-33.

[130] **Tajima Y, Moore MA, Soares V, Ono M, Kissel H, Besmer P.** Consequences of exclusive expression in vivo of Kit-ligand lacking the major proteolytic cleavage site. Proc Natl Acad Sci U S A. 1998;95(20):11903-8.

[131] **Ashman LK.** The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol. 1999;31(10):1037-51.

[132] **Tajima Y, Huang EJ, Vosseller K, Ono M, Moore MA, Besmer P.** Role of dimerization of the membrane-associated growth factor kit ligand in juxtacrine signaling: the Sl17H mutation affects dimerization and stability-phenotypes in hematopoiesis. J Exp Med. 1998;187(9):1451-61.

[133] **Besmer P, Murphy JE, George PC, Qiu FH, Bergold PJ, Lederman L, et al.** A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature. 1986;320(6061):415-21.

[134] **Galli SJ, Zsebo KM, Geissler EN.** The kit ligand, stem cell factor. Adv Immunol. 1994;551-96.

[135] **Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, et al.** Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6(11):3341-51.

[136] **Qiu FH, Ray P, Brown K, Barker PE, Jhanwar S, Ruddle FH, et al.** Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family--oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. EMBO J. 1988;7(4):1003-11.

[137] **Gokkel E, Grossman Z, Ramot B, Yarden Y, Rechavi G, Givol D.** Structural organization of the murine c-kit proto-oncogene. Oncogene. 1992;7(7):1423-9.

[138] **Keshet E, Lyman SD, Williams DE, Anderson DM, Jenkins NA, Copeland NG, et al.** Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. EMBO J. 1991;10(9):2425-35.

[139] **Torihashi S, Ward SM, Nishikawa S, Nishi K, Kobayashi S, Sanders KM.** c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res. 1995;280(1):97-111.

[140] **Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A.** W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature. 1995;373(6512):347-9.

[141] **Natali PG, Nicotra MR, Sures I, Santoro E, Bigotti A, Ullrich A.** Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. Cancer Res. 1992;52(22):6139-43.

[142] **Lammie A, Drobnjak M, Gerald W, Saad A, Cote R, Cordon-Cardo C.** Expression of c-kit and kit ligand proteins in normal human tissues. J Histochem Cytochem. 1994;42(11):1417-25.

[143] **Ma Y, Longley BJ, Wang X, Blount JL, Langley K, Caughey GH.** Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms. J Invest Dermatol. 1999;112(2):165-70.

[144] **Majumder S, Brown K, Qiu FH, Besmer P.** c-kit protein, a transmembrane kinase: identification in tissues and characterization. Mol Cell Biol. 1988;8(11):4896-903.

[145] **Zhang N, Threadgill DW, Womack JE.** Synteny mapping in the bovine: genes from human chromosome 4. Genomics. 1992;14(1):131-6.

[146] **Sakurai M, Zhou JH, Ohtaki M, Itoh T, Murakami Y, Yasue H.** Assignment of c-KIT gene to swine chromosome 8p12-p21 by fluorescence in situ hybridization. Mamm Genome. 1996;7(5):397.

[147] **Sasaki E, Okamura H, Chikamune T, Kanai Y, Watanabe M, Naito M, et al.** Cloning and expression of the chicken c-kit proto-oncogene. Gene. 1993;128(2):257-61.

[148] **Baker CV, Sharpe CR, Torpey NP, Heasman J, Wylie CC.** A Xenopus c-kit-related receptor tyrosine kinase expressed in migrating stem cells of the lateral line system. Mech Dev. 1995;50(2-3):217-28.

[149] **Parichy DM, Rawls JF, Pratt SJ, Whitfield TT, Johnson SL.** Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. Development. 1999;126(15):3425-36.

[150] **Katzenback BA, Belosevic M.** Molecular and functional characterization of kita and kitla of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2009;33(11):1165-75.

[151] **Mellgren EM, Johnson SL.** kitb, a second zebrafish ortholog of mouse Kit. Dev Genes Evol. 2005;215(9):470-77.

[152] **Ullrich A, Schlessinger J.** Signal transduction by receptors with tyrosine kinase activity. Cell. 1990;61(2):203-12.

[153] **Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, et al.** Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood. 1993;82(4):1110-9.

[154] **Fantl WJ, Johnson DE, Williams LT.** Signalling by receptor tyrosine kinases. Annu Rev Biochem. 1993;62453-81.

[155] **Ratajczak J, Machalinski B, Majka M, Kijowski J, Marlicz W, Rozmyslowicz T, et al.** Evidence that human haematopoietic stem cells (HSC) do not reside within the $CD34^{+}KIT^{-}$ cell population. Ann Transplant. 1999;4(1):22-30.

[156] **Cairns LA, Moroni E, Levantini E, Giorgetti A, Klinger FG, Ronzoni S, et al.** Kit regulatory elements required for expression in developing hematopoietic and germ cell lineages. Blood. 2003;102(12):3954-62.

[157] **Ikuta K, Weissman IL.** Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A. 1992;89(4):1502-6.

[158] **Cerisoli F, Cassinelli L, Lamorte G, Citterio S, Bertolotti F, Magli MC, et al.** Green fluorescent protein transgene driven by Kit regulatory sequences is expressed in hematopoietic stem cells. Haematologica. 2009;94(3):318-25.

[159] **Okayama Y, Kawakami T.** Development, migration, and survival of mast cells. Immunol Res. 2006;34(2):97-115.

[160] **Cole SR, Aylett GW, Harvey NL, Cambareri AC, Ashman LK.** Increased expression of c-Kit or its ligand Steel Factor is not a common feature of adult acute myeloid leukaemia. Leukemia. 1996;10(2):288-96.

[161] **Levesque JP, Hendy J, Winkler IG, Takamatsu Y, Simmons PJ.** Granulocyte colony-stimulating factor induces the release in the bone marrow of proteases that cleave c-KIT receptor (CD117) from the surface of hematopoietic progenitor cells. Exp Hematol. 2003;31(2):109-17.

[162] **Broudy VC, Lin NL, Sabath DF.** The fifth immunoglobulin-like domain of the Kit receptor is required for proteolytic cleavage from the cell surface. Cytokine. 2001;15(4):188-95.

[163] **Turner AM, Bennett LG, Lin NL, Wypych J, Bartley TD, Hunt RW, et al.** Identification and characterization of a soluble c-kit receptor produced by human hematopoietic cell lines. Blood. 1995;85(8):2052-8.

[164] **Yee NS, Langen H, Besmer P.** Mechanism of kit ligand, phorbol ester, and calcium-induced down-regulation of c-kit receptors in mast cells. J Biol Chem. 1993;268(19):14189-201.

[165] **Broudy VC, Kovach NL, Bennett LG, Lin N, Jacobsen FW, Kidd PG.** Human umbilical vein endothelial cells display high-affinity c-kit receptors and produce a soluble form of the c-kit receptor. Blood. 1994;83(8):2145-52.

[166] **Wypych J, Bennett LG, Schwartz MG, Clogston CL, Lu HS, Broudy VC, et al.** Soluble kit receptor in human serum. Blood. 1995;85(1):66-73.

[167] **Dahlen DD, Lin NL, Liu YC, Broudy VC.** Soluble Kit receptor blocks stem cell factor bioactivity *in vitro*. Leuk Res. 2001;25(5):413-21.

[168] **Reith AD, Ellis C, Lyman SD, Anderson DM, Williams DE, Bernstein A, et al.** Signal transduction by normal isoforms and W mutant variants of the Kit receptor tyrosine kinase. EMBO J. 1991;10(9):2451-9.

[169] **Hayashi S, Kunisada T, Ogawa M, Yamaguchi K, Nishikawa S.** Exon skipping by mutation of an authentic splice site of c-kit gene in *W/W* mouse. Nucleic Acids Res. 1991;19(6):1267-71.

[170] **Young SM, Cambareri AC, Odell A, Geary SM, Ashman LK.** Early myeloid cells expressing c-KIT isoforms differ in signal transduction, survival and chemotactic responses to Stem Cell Factor. Cell Signal. 2007;19(12):2572-81.

[171] **Crosier PS, Ricciardi ST, Hall LR, Vitas MR, Clark SC, Crosier KE.** Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia. Blood. 1993;82(4):1151-8.

[172] **Jiang X, Gurel O, Mendiaz EA, Stearns GW, Clogston CL, Lu HS, et al.** Structure of the active core of human stem cell factor and analysis of binding to its receptor kit. EMBO J. 2000;19(13):3192-203.

[173] **Philo JS, Wen J, Wypych J, Schwartz MG, Mendiaz EA, Langley KE.** Human stem cell factor dimer forms a complex with two molecules of the extracellular domain of its receptor, Kit. J Biol Chem. 1996;271(12):6895-902.

[174] **Broudy VC.** Stem cell factor and hematopoiesis. Blood. 1997;90(4):1345-64.

[175] **Blechman JM, Lev S, Brizzi MF, Leitner O, Pegoraro L, Givol D, et al.** Soluble c-kit proteins and antireceptor monoclonal antibodies confine the binding site of the stem cell factor. J Biol Chem. 1993;268(6):4399-406.

[176] **Lev S, Blechman J, Nishikawa S, Givol D, Yarden Y.** Interspecies molecular chimeras of kit help define the binding site of the stem cell factor. Mol Cell Biol. 1993;13(4):2224-34.

[177] **Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J.** Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell. 2007;130(2):323-34.

[178] **Narhi LO, Wypych J, Li T, Langley KE, Arakawa T.** Changes in conformation and stability upon SCF/sKit complex formation. J Protein Chem. 1998;17(5):387-96.

[179] **Linnekin D.** Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol. 1999;31(10):1053-74.

[180] **Roskoski R, Jr.** Signaling by Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun. 2005;337(1):1-13.

[181] **Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C.** Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. Clin Cancer Res. 2008;14(7):1926-30.

[182] **Mauduit C, Hamamah S, Benahmed M.** Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update. 1999;5(5):535-45.

[183] **Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, et al.** The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. Dev Suppl. 1993125-37.

[184] **Reid K, Nishikawa S, Bartlett PF, Murphy M.** Steel factor directs melanocyte development in vitro through selective regulation of the number of c-kit^{+} progenitors. Dev Biol. 1995;169(2):568-79.

[185] **Wehrle-Haller B, Weston JA.** Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. Development. 1995;121(3):731-42.

[186] **Yee NS, Paek I, Besmer P.** Role of kit-ligand in proliferation and suppression of apoptosis in mast cells: basis for radiosensitivity of *white* spotting and *steel* mutant mice. J Exp Med. 1994;179(6):1777-87.

[187] **Serve H, Yee NS, Stella G, Sepp-Lorenzino L, Tan JC, Besmer P.** Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. EMBO J. 1995;14(3):473-83.

[188] **Wershil BK, Tsai M, Geissler EN, Zsebo KM, Galli SJ.** The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation *in vivo*. Evidence that signaling through the c-kit receptor can induce expression of cellular function. J Exp Med. 1992;175(1):245-55.

[189] **Ogawa M, Matsuzaki Y, Nishikawa S, Hayashi S, Kunisada T, Sudo T, et al.** Expression and function of c-kit in hemopoietic progenitor cells. J Exp Med. 1991;174(1):63-71.

[190] **Keller JR, Ortiz M, Ruscetti FW.** Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. Blood. 1995;86(5):1757-64.

[191] **Thoren LA, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, et al.** Kit regulates maintenance of quiescent hematopoietic stem cells. J Immunol. 2008;180(4):2045-53.

[192] **Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, et al.** Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver--conditioned medium. Cell. 1990;63(1):195-201.

[193] **Bernstein ID, Andrews RG, Zsebo KM.** Recombinant human stem cell factor enhances the formation of colonies by $CD34^+$ and $CD34^+lin^-$ cells, and the generation of colony-forming cell progeny from $CD34^+lin^-$ cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood. 1991;77(11):2316-21.

[194] **Mantel C, Hendrie P, Broxmeyer HE.** Steel factor regulates cell cycle asymmetry. Stem Cells. 2001;19(6):483-91.

[195] **Okumura N, Tsuji K, Ebihara Y, Tanaka I, Sawai N, Koike K, et al.** Chemotactic and chemokinetic activities of stem cell factor on murine hematopoietic progenitor cells. Blood. 1996;87(10):4100-8.

[196] **Bedell MA, Mahakali Zama A.** Genetic analysis of Kit ligand functions during mouse spermatogenesis. J Androl. 2004;25(2):188-99.

[197] **Wehrle-Haller B.** The role of Kit-ligand in melanocyte development and epidermal homeostasis. Pigment Cell Res. 2003;16(3):287-96.

[198] **Tanaka A, Arai K, Kitamura Y, Matsuda H.** Matrix metalloproteinase-9 production, a newly identified function of mast cell progenitors, is downregulated by c-kit receptor activation. Blood. 1999;94(7):2390-5.

[199] **Lukacs NW, Kunkel SL, Strieter RM, Evanoff HL, Kunkel RG, Key ML, et al.** The role of stem cell factor (c-kit ligand) and inflammatory cytokines in pulmonary mast cell activation. Blood. 1996;87(6):2262-8.

[200] **Reber L, Da Silva CA, Frossard N.** Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur J Pharmacol. 2006;533(1-3):327-40.

[201] **Hultman KA, Bahary N, Zon LI, Johnson SL.** Gene Duplication of the zebrafish kit ligand and partitioning of melanocyte development functions to kit ligand a. PLoS Genet. 2007;3(1):e17.

[202] **Yao K, Ge W.** Kit system in the zebrafish ovary: evidence for functional divergence of two isoforms of kit (kita and kitb) and kit ligand (kitlga and kitlgb) during folliculogenesis. Biol Reprod. 2010;82(6):1216-26.

[203] **Cooper CD, Linbo TH, Raible DW.** Kit and foxd3 genetically interact to regulate melanophore survival in zebrafish. Dev Dyn. 2009;238(4):875-86.

[204] **Mellgren EM, Johnson SL.** A requirement for kit in embryonic zebrafish melanocyte differentiation is revealed by melanoblast delay. Dev Genes Evol. 2004;214(10):493-502.

[205] **Rawls JF, Johnson SL.** Requirements for the kit receptor tyrosine kinase during regeneration of zebrafish fin melanocytes. Development. 2001;128(11):1943-9.

[206] **Yamada T, Okauchi M, Araki K.** Origin of adult-type pigment cells forming the asymmetric pigment pattern, in Japanese flounder (*Paralichthys olivaceus*). Dev Dyn. 2010;239(12):3147-62.

[207] **Mills MG, Nuckels RJ, Parichy DM.** Deconstructing evolution of adult phenotypes: genetic analyses of kit reveal homology and evolutionary novelty during adult pigment pattern development of Danio fishes. Development. 2007;134(6):1081-90.

[208] **Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F, et al.** Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, p cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. J Immunol. 1983;131(1):282-7.

[209] **Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, et al.** Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell. 1986;47(1):3-10.

[210] **Rennick DM, Lee FD, Yokota T, Arai KI, Cantor H, Nabel GJ.** A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: multiple activities of interleukin 3. J Immunol. 1985;134(2):910-4.

[211] **Burger H, van Leen RW, Dorssers LC, Persoon NL, Lemson PJ, Wagemaker G.** Species specificity of human interleukin-3 demonstrated by cloning and expression of the homologous rhesus monkey (*Macaca mulatta*) gene. Blood. 1990;76(11):2229-34.

[212] **Yokota T, Lee F, Rennick D, Hall C, Arai N, Mosmann T, et al.** Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in monkey cells. Proc Natl Acad Sci U S A. 1984;81(4):1070-4.

[213] **del CEM, van Someren GD, van der Velde I, van Bekkum DW, Valerio D, Noteboom JL, et al.** Cloning, biological characterization and high-level expression of rat interleukin-3 using recombinant adenovirus: description of a new splicing variant. Gene. 1998;211(1):151-8.

[214] **Mwangi SM, Logan-Henfrey L, McInnes C, Mertens B.** Cloning of the bovine interleukin-3-encoding cDNA. Gene. 1995;162(2):309-12.

[215] **McInnes C, Haig D, Logan M.** The cloning and expression of the gene for ovine interleukin-3 (multi-CSF) and a comparison of the *in vitro* hematopoietic activity of ovine IL-3 with ovine GM-CSF and human M-CSF. Exp Hematol. 1993;21(12):1528-34.

[216] **Barreda DR, Hanington PC, Belosevic M.** Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol. 2004;28(5):509-54.

[217] **Martinez-Moczygemba M, Huston DP.** Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. J Allergy Clin Immunol. 2003;112(4):653-65; quiz 66.

[218] **Scott CL, Begley CG.** The beta common chain (beta c) of the granulocyte macrophage-colony stimulating factor, interleukin-3 and interleukin-5 receptors. Int J Biochem Cell Biol. 1999;31(10):1011-5.

[219] **Le Beau MM, Epstein ND, O'Brien SJ, Nienhuis AW, Yang YC, Clark SC, et al.** The interleukin 3 gene is located on human chromosome 5 and is deleted in myeloid leukemias with a deletion of 5q. Proc Natl Acad Sci U S A. 1987;84(16):5913-7.

[220] **Webb GC, Lee JS, Campbell HD, Young IG.** The genes for interleukins 3 and 5 map to the same locus on mouse chromosome 11. Cytogenet Cell Genet. 1989;50(2-3):107-10.

[221] **Fung MC, Hapel AJ, Ymer S, Cohen DR, Johnson RM, Campbell HD, et al.** Molecular cloning of cDNA for murine interleukin-3. Nature. 1984;307(5948):233-7.

[222] **Rozwarski DA, Gronenborn AM, Clore GM, Bazan JF, Bohm A, Wlodawer A, et al.** Structural comparisons among the short-chain helical cytokines. Structure. 1994;2(3):159-73.

[223] **Yao S, Young IG, Norton RS, Murphy JM.** Murine interleukin-3: structure, dynamics, and conformational heterogeneity in solution. Biochemistry. 2011;50(13):2464-77.

[224] **van Leeuwen BH, Martinson ME, Webb GC, Young IG.** Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. Blood. 1989;73(5):1142-8.

[225] **Kitamura T, Sato N, Arai K, Miyajima A.** Expression cloning of the human IL-3 receptor cDNA reveals a shared beta subunit for the human IL-3 and GM-CSF receptors. Cell. 1991;66(6):1165-74.

[226] **Milatovich A, Kitamura T, Miyajima A, Francke U.** Gene for the alpha-subunit of the human interleukin-3 receptor (IL3RA) localized to the X-Y pseudoautosomal region. Am J Hum Genet. 1993;53(5):1146-53.

[227] **Kremer E, Baker E, D'Andrea RJ, Slim R, Phillips H, Moretti PA, et al.** A cytokine receptor gene cluster in the X-Y pseudoautosomal region? Blood. 1993;82(1):22-8.

[228] **Miyajima I, Levitt L, Hara T, Bedell MA, Copeland NG, Jenkins NA, et al.** The murine interleukin-3 receptor alpha subunit gene: chromosomal localization, genomic structure, and promoter function. Blood. 1995;85(5):1246-53.

[229] **Chen J, Olsen J, Ford S, Mirza S, Walker A, Murphy JM, et al.** A new isoform of interleukin-3 receptor {alpha} with novel differentiation activity and high affinity binding mode. J Biol Chem. 2009;284(9):5763-73.

[230] **Mirza S, Chen J, Wen B, Ewens CL, Dai J, Murphy JM, et al.** Two modes of beta-receptor recognition are mediated by distinct epitopes on mouse and human interleukin-3. J Biol Chem. 2010;285(29):22370-81.

[231] **Shen Y, Baker E, Callen DF, Sutherland GR, Willson TA, Rakar S, et al.** Localization of the human GM-CSF receptor beta chain gene (CSF2RB) to chromosome 22q12.2-->q13.1. Cytogenet Cell Genet. 1992;61(3):175-7.

[232] **Gorman DM, Itoh N, Jenkins NA, Gilbert DJ, Copeland NG, Miyajima A.** Chromosomal localization and organization of the murine genes encoding the beta subunits (AIC2A and AIC2B) of the interleukin 3, granulocyte/macrophage colony-stimulating factor, and interleukin 5 receptors. J Biol Chem. 1992;267(22):15842-8.

[233] **Gale RE, Freeburn RW, Khwaja A, Chopra R, Linch DC.** A truncated isoform of the human beta chain common to the receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3), and IL-5 with increased mRNA expression in some patients with acute leukemia. Blood. 1998;91(1):54-63.

[234] **Carr PD, Gustin SE, Church AP, Murphy JM, Ford SC, Mann DA, et al.** Structure of the complete extracellular domain of the common beta subunit of the human GM-CSF, IL-3, and IL-5 receptors reveals a novel dimer configuration. Cell. 2001;104(2):291-300.

[235] **Dey R, Ji K, Liu Z, Chen L.** A cytokine-cytokine interaction in the assembly of higher-order structure and activation of the interleukine-3:receptor complex. PLoS One. 2009;4(4):e5188.

[236] **Otsuka T, Miyajima A, Brown N, Otsu K, Abrams J, Saeland S, et al.** Isolation and characterization of an expressible cDNA encoding human IL-3. Induction of IL-3 mRNA in human T cell clones. J Immunol. 1988;140(7):2288-95.

[237] **Spivak JL, Smith RR, Ihle JN.** Interleukin 3 promotes the in vitro proliferation of murine pluripotent hematopoietic stem cells. J Clin Invest. 1985;76(4):1613-21.

[238] **Quesenberry PJ, Ihle JN, McGrath E.** The effect of interleukin 3 and GM-CSA-2 on megakaryocyte and myeloid clonal colony formation. Blood. 1985;65(1):214-7.

[239] **Metcalf D, Begley CG, Johnson GR, Nicola NA, Lopez AF, Williamson DJ.** Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hematopoiesis in normal adult mice. Blood. 1986;68(1):46-57.

[240] **Wagemaker G, van Gils FC, Burger H, Dorssers LC, van Leen RW, Persoon NL, et al.** Highly increased production of bone marrow-derived blood cells by administration of homologous interleukin-3 to rhesus monkeys. Blood. 1990;76(11):2235-41.

[241] **Nishinakamura R, Nakayama N, Hirabayashi Y, Inoue T, Aud D, McNeil T, et al.** Mice deficient for the IL-3/GM-CSF/IL-5 beta c receptor exhibit lung pathology and impaired immune response, while beta IL3 receptor-deficient mice are normal. Immunity. 1995;2(3):211-22.

[242] **Nishinakamura R, Miyajima A, Mee PJ, Tybulewicz VL, Murray R.** Hematopoiesis in mice lacking the entire granulocyte-macrophage colony-stimulating factor/interleukin-3/interleukin-5 functions. Blood. 1996;88(7):2458-64.

[243] **Gasson JC, Weisbart RH, Kaufman SE, Clark SC, Hewick RM, Wong GG, et al.** Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. Science. 1984;226(4680):1339-42.

[244] **Cantrell MA, Anderson D, Cerretti DP, Price V, McKereghan K, Tushinski RJ, et al.** Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. Proc Natl Acad Sci U S A. 1985;82(18):6250-4.

[245] **Gough NM, Gough J, Metcalf D, Kelso A, Grail D, Nicola NA, et al.** Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. Nature. 1984;309(5971):763-7.

[246] **Gough NM, Metcalf D, Gough J, Grail D, Dunn AR.** Structure and expression of the mRNA for murine granulocyte-macrophage colony stimulating factor. EMBO J. 1985;4(3):645-53.

[247] **Maliszewski CR, Schoenborn MA, Cerretti DP, Wignall JM, Picha KS, Cosman D, et al.** Bovine GM-CSF: molecular cloning and biological activity of the recombinant protein. Mol Immunol. 1988;25(9):843-50.

[248] **McInnes CJ, Haig DM.** Cloning and expression of a cDNA encoding ovine granulocyte-macrophage colony-stimulating factor. Gene. 1991;105(2):275-9.

[249] **Inumaru S, Takamatsu H.** cDNA cloning of porcine granulocyte-macrophage colony-stimulating factor. Immunol Cell Biol. 1995;73(5):474-6.

[250] **Wu HL, Chen PJ, Lin HK, Lee RS, Lin HL, Liu CJ, et al.** Molecular cloning and expression of woodchuck granulocyte-macrophage colony stimulating factor. J Med Virol. 2001;65(3):567-75.

[251] **Stanley E, Metcalf D, Sobieszczuk P, Gough NM, Dunn AR.** The structure and expression of the murine gene encoding granulocyte-macrophage colony stimulating factor: evidence for utilisation of alternative promoters. EMBO J. 1985;4(10):2569-73.

[252] **Miyatake S, Otsuka T, Yokota T, Lee F, Arai K.** Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: comparison of the mouse and human genes. EMBO J. 1985;4(10):2561-8.

[253] **Barlow DP, Bucan M, Lehrach H, Hogan BL, Gough NM.** Close genetic and physical linkage between the murine haemopoietic growth factor genes GM-CSF and Multi-CSF (IL3). EMBO J. 1987;6(3):617-23.

[254] **Gough NM, Gearing DP, Nicola NA, Baker E, Pritchard M, Callen DF, et al.** Localization of the human GM-CSF receptor gene to the X-Y pseudoautosomal region. Nature. 1990;345(6277):734-6.

[255] **Rappold G, Willson TA, Henke A, Gough NM.** Arrangement and localization of the human GM-CSF receptor alpha chain gene CSF2RA within the X-Y pseudoautosomal region. Genomics. 1992;14(2):455-61.

[256] **Disteche CM, Brannan CI, Larsen A, Adler DA, Schorderet DF, Gearing D, et al.** The human pseudoautosomal GM-CSF receptor alpha subunit gene is autosomal in mouse. Nat Genet. 1992;1(5):333-6.

[257] **Gearing DP, King JA, Gough NM, Nicola NA.** Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO J. 1989;8(12):3667-76.

[258] **Mirza S, Walker A, Chen J, Murphy JM, Young IG.** The Ig-like domain of human GM-CSF receptor alpha plays a critical role in cytokine binding and receptor activation. Biochem J. 2010;426(3):307-17.

[259] **Hansen G, Hercus TR, McClure BJ, Stomski FC, Dottore M, Powell J, et al.** The structure of the GM-CSF receptor complex reveals a distinct mode of cytokine receptor activation. Cell. 2008;134(3):496-507.

[260] **Hercus TR, Thomas D, Guthridge MA, Ekert PG, King-Scott J, Parker MW, et al.** The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease. Blood. 2009;114(7):1289-98.

[261] **Broudy VC, Kaushansky K, Segal GM, Harlan JM, Adamson JW.** Tumor necrosis factor type alpha stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. Proc Natl Acad Sci U S A. 1986;83(19):7467-71.

[262] **Munker R, Gasson J, Ogawa M, Koeffler HP.** Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. Nature. 1986;323(6083):79-82.

[263] **Kimura A, Rieger MA, Simone JM, Chen W, Wickre MC, Zhu BM, et al.** The transcription factors STAT5A/B regulate GM-CSF-mediated granulopoiesis. Blood. 2009;114(21):4721-8.

[264] **Iwasaki-Arai J, Iwasaki H, Miyamoto T, Watanabe S, Akashi K.** Enforced granulocyte/macrophage colony-stimulating factor signals do not support lymphopoiesis, but instruct lymphoid to myelomonocytic lineage conversion. J Exp Med. 2003;197(10):1311-22.

[265] **Gomez-Cambronero J, Horn J, Paul CC, Baumann MA.** Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. J Immunol. 2003;171(12):6846-55.

[266] **Khajah M, Millen B, Cara DC, Waterhouse C, McCafferty DM.** Granulocyte-macrophage colony-stimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes *in vivo*. J Leukoc Biol. 2011;89(6):945-53.

[267] **Fossati G, Mazzucchelli I, Gritti D, Ricevuti G, Edwards SW, Moulding DA, et al.** *In vitro* effects of GM-CSF on mature peripheral blood neutrophils. Int J Mol Med. 1998;1(6):943-51.

[268] **Watowich SS, Liu YJ.** Mechanisms regulating dendritic cell specification and development. Immunol Rev. 2010;238(1):76-92.

[269] **Schmid MA, Kingston D, Boddupalli S, Manz MG.** Instructive cytokine signals in dendritic cell lineage commitment. Immunol Rev. 2010;234(1):32-44.

[270] **Laiosa CV, Stadtfeld M, Graf T.** Determinants of lymphoid-myeloid lineage diversification. Annu Rev Immunol. 2006;24705-38.

[271] **Iwasaki H, Akashi K.** Myeloid lineage commitment from the hematopoietic stem cell. Immunity. 2007;26(6):726-40.

[272] **Rosenbauer F, Tenen DG.** Transcription factors in myeloid development: balancing differentiation with transformation. Nat Rev Immunol. 2007;7(2):105-17.

[273] **Hanington PC, Tam J, Katzenback BA, Hitchen SJ, Barreda DR, Belosevic M.** Development of macrophages of cyprinid fish. Dev Comp Immunol. 2009;33(4):411-29.

[274] **Sieweke MH, Tekotte H, Frampton J, Graf T.** MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. Cell. 1996;85(1):49-60.

[275] **Kajihara M, Kawauchi S, Kobayashi M, Ogino H, Takahashi S, Yasuda K.** Isolation, characterization, and expression analysis of zebrafish large Mafs. J Biochem. 2001;129(1):139-46.

[276] **Landschulz WH, Johnson PF, McKnight SL.** The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science. 1988;240(4860):1759-64.

[277] **Lekstrom-Himes J, Xanthopoulos KG.** Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem. 1998;273(44):28545-8.

[278] **Lyons SE, Shue BC, Lei L, Oates AC, Zon LI, Liu PP.** Molecular cloning, genetic mapping, and expression analysis of four zebrafish c/ebp genes. Gene. 2001;281(1-2):43-51.

[279] **Fujiki K, Gerwick L, Bayne CJ, Mitchell L, Gauley J, Bols N, et al.** Molecular cloning and characterization of rainbow trout (*Oncorhynchus mykiss*) CCAAT/enhancer binding protein beta. Immunogenetics. 2003;55(4):253-61.

[280] **Tucker CS, Hirono I, Aoki T.** Molecular cloning and expression of CCAAT/enhancer binding proteins in Japanese flounder *Paralichthys olivaceus*. Dev Comp Immunol. 2002;26(3):271-82.

[281] **Hikima J, Ohtani M, Kondo H, Hirono I, Jung TS, Aoki T.** Characterization and gene expression of transcription factors, PU.1 and C/EBPalpha driving transcription from the tumor necrosis factor alpha promoter in Japanese flounder, *Paralichthys olivaceus*. Dev Comp Immunol. 2011;35(3):304-13.

[282] **Traver D, Miyamoto T, Christensen J, Iwasaki-Arai J, Akashi K, Weissman IL.** Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. Blood. 2001;98(3):627-35.

[283] **Hohaus S, Petrovick MS, Voso MT, Sun Z, Zhang DE, Tenen DG.** PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. Mol Cell Biol. 1995;15(10):5830-45.

[284] **Heath V, Suh HC, Holman M, Renn K, Gooya JM, Parkin S, et al.** C/EBPalpha deficiency results in hyperproliferation of hematopoietic progenitor cells and disrupts macrophage development *in vitro* and *in vivo*. Blood. 2004;104(6):1639-47.

[285] **Friedman AD.** C/EBPalpha induces PU.1 and interacts with AP-1 and NF-kappaB to regulate myeloid development. Blood Cells Mol Dis. 2007;39(3):340-3.

[286] **Zhang P, Iwasaki-Arai J, Iwasaki H, Fenyus ML, Dayaram T, Owens BM, et al.** Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha. Immunity. 2004;21(6):853-63.

[287] **Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, et al.** Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell. 2006;126(4):755-66.

[288] **Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG.** CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol. 1998;18(7):4301-14.

[289] **Ford AM, Bennett CA, Healy LE, Towatari M, Greaves MF, Enver T.** Regulation of the myeloperoxidase enhancer binding proteins Pu1, C-EBP alpha,

-beta, and -delta during granulocyte-lineage specification. Proc Natl Acad Sci U S A. 1996;93(20):10838-43.

[290] **Oelgeschlager M, Nuchprayoon I, Luscher B, Friedman AD.** C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. Mol Cell Biol. 1996;16(9):4717-25.

[291] **Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG.** PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. Blood. 1996;88(4):1234-47.

[292] **Liu TX, Rhodes J, Deng M, Hsu K, Radomska HS, Kanki JP, et al.** Dominant-interfering C/EBPalpha stimulates primitive erythropoiesis in zebrafish. Exp Hematol. 2007;35(2):230-9.

[293] **Yuan H, Zhou J, Deng M, Zhang Y, Chen Y, Jin Y, et al.** Sumoylation of CCAAT/enhancer-binding protein alpha promotes the biased primitive hematopoiesis of zebrafish. Blood. 2011;117(26):7014-20.

[294] **Barreda DR, Hanington PC, Walsh CK, Wong P, Belosevic M.** Differentially expressed genes that encode potential markers of goldfish macrophage development in vitro. Dev Comp Immunol. 2004;28(7-8):727-46.

[295] **Burda P, Laslo P, Stopka T.** The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. Leukemia. 2010;24(7):1249-57.

[296] **Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA.** The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell. 1990;61(1):113-24.

[297] **Kim HG, de Guzman CG, Swindle CS, Cotta CV, Gartland L, Scott EW, et al.** The ETS family transcription factor PU.1 is necessary for the maintenance of fetal liver hematopoietic stem cells. Blood. 2004;104(13):3894-900.

[298] **Iwasaki H, Somoza C, Shigematsu H, Duprez EA, Iwasaki-Arai J, Mizuno S, et al.** Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. Blood. 2005;106(5):1590-600.

[299] **Dakic A, Metcalf D, Di Rago L, Mifsud S, Wu L, Nutt SL.** PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. J Exp Med. 2005;201(9):1487-502.

[300] **McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, et al.** Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. 1996;15(20):5647-58.

[301] **Scott EW, Simon MC, Anastasi J, Singh H.** Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science. 1994;265(5178):1573-7.

[302] **Nutt SL, Metcalf D, D'Amico A, Polli M, Wu L.** Dynamic regulation of PU.1 expression in multipotent hematopoietic progenitors. J Exp Med. 2005;201(2):221-31.

[303] **Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, et al.** PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. Blood. 2000;96(8):2641-8.

[304] **Rekhtman N, Radparvar F, Evans T, Skoultchi AI.** Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. Genes Dev. 1999;13(11):1398-411.

[305] **Xie Y, Chen C, Hume DA.** Transcriptional regulation of *c-fms* gene expression. Cell Biochem Biophys. 2001;34(1):1-16.

[306] **Tagoh H, Himes R, Clarke D, Leenen PJ, Riggs AD, Hume D, et al.** Transcription factor complex formation and chromatin fine structure alterations at the murine *c-fms* (CSF-1 receptor) locus during maturation of myeloid precursor cells. Genes Dev. 2002;16(13):1721-37.

[307] **Gangenahalli GU, Gupta P, Saluja D, Verma YK, Kishore V, Chandra R, et al.** Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. Stem Cells Dev. 2005;14(2):140-52.

[308] **Ito Y, Seto Y, Brannan CI, Copeland NG, Jenkins NA, Fukunaga R, et al.** Structural analysis of the functional gene and pseudogene encoding the murine granulocyte colony-stimulating-factor receptor. Eur J Biochem. 1994;220(3):881-91.

[309] **Lieschke GJ, Oates AC, Paw BH, Thompson MA, Hall NE, Ward AC, et al.** Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. Dev Biol. 2002;246(2):274-95.

[310] **Ward AC, McPhee DO, Condron MM, Varma S, Cody SH, Onnebo SM, et al.** The zebrafish spi1 promoter drives myeloid-specific expression in stable transgenic fish. Blood. 2003;102(9):3238-40.

[311] **Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, et al.** The pu.1 promoter drives myeloid gene expression in zebrafish. Blood. 2004;104(5):1291-7.

[312] **Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, et al.** Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. Dev Cell. 2005;8(1):97-108.

[313] **Galloway JL, Wingert RA, Thisse C, Thisse B, Zon LI.** Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. Dev Cell. 2005;8(1):109-16.

[314] **Zakrzewska A, Cui C, Stockhammer OW, Benard EL, Spaink HP, Meijer AH.** Macrophage-specific gene functions in Spi1-directed innate immunity. Blood. 2010;116(3):e1-11.

[315] **Bukrinsky A, Griffin KJ, Zhao Y, Lin S, Banerjee U.** Essential role of spi-1-like (spi-1l) in zebrafish myeloid cell differentiation. Blood. 2009;113(9):2038-46.

[316] **Takahashi K.** Development and differentiation of macrophages and related cells: historical review and current concepts. Journal of Clinical and Experimental Hematopathology. 2001;411-33.

[317] **Auffray C, Sieweke MH, Geissmann F.** Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27669-92.

[318] **Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K.** Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61.

[319] **Ganassin RC, Bols NC.** Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. Fish Shellfish Immunol. 1998;8457-76.

[320] **Stafford JL, McLauchlan PE, Secombes CJ, Ellis AE, Belosevic M.** Generation of primary monocyte-like cultures from rainbow trout head kidney leukocytes. Dev Comp Immunol. 2001;25(5-6):447-59.

[321] **Stachura DL, Reyes JR, Bartunek P, Paw BH, Zon LI, Traver D.** Zebrafish kidney stromal cell lines support multilineage hematopoiesis. Blood. 2009;114(2):279-89.

[322] **Stachura DL, Svoboda O, Lau RP, Balla KM, Zon LI, Bartunek P, et al.** Clonal analysis of hematopoietic progenitor cells in the zebrafish. Blood. 2011;118(5):1274-82.

[323] **Bennett CM, Kanki JP, Rhodes J, Liu TX, Paw BH, Kieran MW, et al.** Myelopoiesis in the zebrafish, *Danio rerio*. Blood. 2001;98(3):643-51.

[324] **Crowhurst MO, Layton JE, Lieschke GJ.** Developmental biology of zebrafish myeloid cells. Int J Dev Biol. 2002;46(4):483-92.

[325] **Zuasti A, Ferrer C.** Haemopoiesis in the head kidney of *Sparus auratus*. Arch Histol Cytol. 1989;52(3):249-55.

[326] **Abdel-Aziz el SH, Abdu SB, Ali Tel S, Fouad HF.** Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. Fish Physiol Biochem. 2010;36(3):323-36.

[327] **Wittamer V, Bertrand JY, Gutschow PW, Traver D.** Characterization of the mononuclear phagocyte system in zebrafish. Blood. 2011;117(26):7126-35.

[328] **Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P.** Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. PLoS Biol. 2010;8(12):e1000562.

[329] **Grabher C, Cliffe A, Miura K, Hayflick J, Pepperkock R, Rorth P, et al.** Birth and life of tissue macrophages and their migration in embryogenesis and inflammation in medaka. Journal of Leukocyte Biology. 2007;81263-71.

[330] **Mathias JR, Dodd ME, Walters KB, Yoo SK, Ranheim EA, Huttenlocher A.** Characterization of zebrafish larval inflammatory macrophages. Dev Comp Immunol. 2009;33(11):1212-7.

[331] **Fixe P, Praloran V.** Macrophage colony-stimulating-factor (M-CSF or CSF-1) and its receptor: structure-function relationships. Eur Cytokine Netw. 1997;8(2):125-36.

[332] **Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, et al.** Biology and action of colony--stimulating factor-1. Mol Reprod Dev. 1997;46(1):4-10.

[333] **Guilbert LJ, Stanley ER.** Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 1980;85(1):153-9.

[334] **Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER.** Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell. 1982;28(1):71-81.

[335] **Kawasaki ES, Ladner MB.** Molecular biology of macrophage colony-stimulating factor. Immunol Ser. 1990;49155-76.

[336] **Douglass TG, Driggers L, Zhang JG, Hoa N, Delgado C, Williams CC, et al.** Macrophage colony stimulating factor: not just for macrophages anymore! A gateway into complex biologies. Int Immunopharmacol. 2008;8(10):1354-76.

[337] **Chitu V, Stanley ER.** Colony-stimulating factor-1 in immunity and inflammation. Curr Opin Immunol. 2006;18(1):39-48.

[338] **Borycki A, Lenormund J, Guillier M, Leibovitch SA.** Isolation and characterization of a cDNA clone encoding for rat CSF-1 gene. Post-transcriptional repression occurs in myogenic differentiation. Biochim Biophys Acta. 1993;1174(2):143-52.

[339] **Yoshihara K, Inumaru S, Hirota Y, Momotani E.** Cloning and sequencing of cDNA encoding bovine macrophage colony-stimulating factor (bM-CSF) and expression of recombinant bM-CSF using baculovirus. Vet Immunol Immunopathol. 1998;63(4):381-91.

[340] **Garceau V, Smith J, Paton IR, Davey M, Fares MA, Sester DP, et al.** Pivotal Advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. J Leukoc Biol. 2010;87(5):753-64.

[341] **Hanington PC, Wang T, Secombes CJ, Belosevic M.** Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[342] **Wang T, Hanington PC, Belosevic M, Secombes CJ.** Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. J Immunol. 2008;181(5):3310-22.

[343] **Ladner MB, Martin GA, Noble JA, Nikoloff DM, Tal R, Kawasaki ES, et al.** Human CSF-1: gene structure and alternative splicing of mRNA precursors. EMBO J. 1987;6(9):2693-8.

[344] **Morris SW, Valentine MB, Shapiro DN, Sublett JE, Deaven LL, Foust JT, et al.** Reassignment of the human CSF1 gene to chromosome 1p13-p21. Blood. 1991;78(8):2013-20.

[345] **Saltman DL, Dolganov GM, Hinton LM, Lovett M.** Reassignment of the human macrophage colony stimulating factor gene to chromosome 1p13-21. Biochem Biophys Res Commun. 1992;182(3):1139-43.

[346] **Gisselbrecht S, Sola B, Fichelson S, Bordereaux D, Tambourin P, Mattei MG, et al.** The murine M-CSF gene is localized on chromosome 3. Blood. 1989;73(6):1742-5.

[347] **Buchberg AM, Jenkins NA, Copeland NG.** Localization of the murine macrophage colony-stimulating factor gene to chromosome 3 using interspecific backcross analysis. Genomics. 1989;5(2):363-7.

[348] **Pandit J, Bohm A, Jancarik J, Halenbeck R, Koths K, Kim SH.** Three-dimensional structure of dimeric human recombinant macrophage colony-stimulating factor. Science. 1992;258(5086):1358-62.

[349] **Shadle PJ, Allen JI, Geier MD, Koths K.** Detection of endogenous macrophage colony-stimulating factor (M-CSF) in human blood. Exp Hematol. 1989;17(2):154-9.

[350] **Praloran V, Gascan H, Papin S, Chevalier S, Trossaert M, Boursier MC.** Inducible production of macrophage colony-stimulating factor (CSF-1) by malignant and normal human T cells. Leukemia. 1990;4(6):411-4.

[351] **Fretier S, Besse A, Delwail A, Garcia M, Morel F, Leprivey-Lorgeot V, et al.** Cyclosporin A inhibition of macrophage colony-stimulating factor (M-CSF) production by activated human T lymphocytes. J Leukoc Biol. 2002;71(2):289-94.

[352] **Hallet MM, Praloran V, Vie H, Peyrat MA, Wong G, Witek-Giannotti J, et al.** Macrophage colony-stimulating factor (CSF-1) gene expression in human T-lymphocyte clones. Blood. 1991;77(4):780-6.

[353] **Horiguchi J, Warren MK, Kufe D.** Expression of the macrophage-specific colony-stimulating factor in human monocytes treated with granulocyte-macrophage colony-stimulating factor. Blood. 1987;69(4):1259-61.

[354] **Oster W, Lindemann A, Horn S, Mertelsmann R, Herrmann F.** Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. Blood. 1987;70(5):1700-3.

[355] **Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, et al.** Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science. 2008;320(5877):807-11.

[356] **Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al.** Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. J Leukoc Biol. 2010;88(3):495-505.

[357] **Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, et al.** IL-34 and M-CSF share the receptor *Fms* but are not identical in biological activity and signal activation. Cell Death Differ. 2010;17(12):1917-27.

[358] **Chen Z, Buki K, Vaaraniemi J, Gu G, Vaananen HK.** The critical role of IL-34 in osteoclastogenesis. PLoS One. 2011;6(4):e18689.

[359] **Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al.** Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330(6005):841-5.

[360] **Groffen J, Heisterkamp N, Spurr N, Dana S, Wasmuth JJ, Stephenson JR.** Chromosomal localization of the human *c-fms* oncogene. Nucleic Acids Res. 1983;11(18):6331-9.

[361] **Roussel MF, Sherr CJ, Barker PE, Ruddle FH.** Molecular cloning of the *c-fms* locus and its assignment to human chromosome 5. J Virol. 1983;48(3):770-3.

[362] **Hoggan MD, Halden NF, Buckler CE, Kozak CA.** Genetic mapping of the mouse *c-fms* proto-oncogene to chromosome 18. J Virol. 1988;62(3):1055-6.

[363] **Hampe A, Shamoon BM, Gobet M, Sherr CJ, Galibert F.** Nucleotide sequence and structural organization of the human FMS proto-oncogene. Oncogene Res. 1989;4(1):9-17.

[364] **Rettenmier CW, Chen JH, Roussel MF, Sherr CJ.** The product of the *c-fms* proto-oncogene: a glycoprotein with associated tyrosine kinase activity. Science. 1985;228(4697):320-2.

[365] **Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER.** The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. 1985;41(3):665-76.

[366] **Pixley FJ, Stanley ER.** CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol. 2004;14(11):628-38.

[367] **Chen X, Liu H, Focia PJ, Shim AH, He X.** Structure of macrophage colony stimulating factor bound to FMS: diverse signaling assemblies of class III receptor tyrosine kinases. Proc Natl Acad Sci U S A. 2008;105(47):18267-72.

[368] **Hamilton JA.** CSF-1 signal transduction. J Leukoc Biol. 1997;62(2):145-55.

[369] **Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, et al.** Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages *in vitro* in response to CSF-1. J Leukoc Biol. 2007;82(1):111-23.

[370] **Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, et al.** The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Letters to Nature. 1990;345442-4.

[371] **Marks SC, Jr., Lane PW.** Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J Hered. 1976;67(1):11-8.

[372] **Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr., Ahmed-Ansari A, Sell KW, Pollard JW, et al.** Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci U S A. 1990;87(12):4828-32.

[373] **Pollard JW, Stanley ER.** Pleiotropic roles for CSF-1 in development defined by the mouse mutation osteopetrotic. Advances in Developmental Biochemistry. 1996;4153-93.

[374] **Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al.** Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood. 2002;99(1):111-20.

[375] **Hanington PC, Hitchen SJ, Beamish LA, Belosevic M.** Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. Fish Shellfish Immunol. 2009;26(1):1-9.

[376] **Grayfer L, Hanington PC, Belosevic M.** Macrophage colony-stimulating factor (CSF-1) induces pro-inflammatory gene expression and enhances antimicrobial responses of goldfish (*Carassius auratus* L.) macrophages. Fish Shellfish Immunol. 2009;26(3):406-13.

[377] **How GF, Venkatesh B, Brenner S.** Conserved linkage between the puffer fish (*Fugu rubripes*) and human genes for platelet-derived growth factor receptor and macrophage colony-stimulating factor receptor. Genome Res. 1996;6(12):1185-91.

[378] **Williams H, Brenner S, Venkatesh B.** Identification and analysis of additional copies of the platelet-derived growth factor receptor and colony stimulating factor 1 receptor genes in fugu. Gene. 2002;295(2):255-64.

[379] **Herbomel P, Thisse B, Thisse C.** Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001;238(2):274-88.

[380] **Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, et al.** Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. Mol Immunol. 2005;42(1):1-8.

[381] **Roca FJ, Sepulcre MA, Lopez-Castejon G, Meseguer J, Mulero V.** The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. Mol Immunol. 2006;43(9):1418-23.

[382] **Barreda DR, Hanington PC, Stafford JL, Belosevic M.** A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[383] **Mulero I, Pilar Sepulcre M, Roca FJ, Meseguer J, Garcia-Ayala A, Mulero V.** Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev Comp Immunol. 2008;32(10):1151-9.

[384] **Braasch I, Salzburger W, Meyer A.** Asymmetric evolution in two fish-specifically duplicated receptor tyrosine kinase paralogons involved in teleost coloration. Mol Biol Evol. 2006;23(6):1192-202.

[385] **Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL.** An orthologue of the kit-related gene *fms* is required for development of neural crest-derived

xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. Development. 2000;127(14):3031-44.

[386] **Parichy DM, Turner JM.** Temporal and cellular requirements for *Fms* signaling during zebrafish adult pigment pattern development. Development. 2003;130(5):817-33.

[387] **Katzenback BA, Belosevic M.** Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney. Dev Comp Immunol. 2009;33(4):601-11.

[388] **Theilgaard-Monch K, Jacobsen LC, Borup R, Rasmussen T, Bjerregaard MD, Nielsen FC, et al.** The transcriptional program of terminal granulocytic differentiation. Blood. 2005;105(4):1785-96.

[389] **Meseguer J, Esteban MA, Garcia Ayala A, Lopez Ruiz A, Agulleiro B.** Granulopoiesis in the head-kidney of the sea bass (*Dicentrarchus labrax* L.): an ultrastructural study. Arch Histol Cytol. 1990;53(3):287-96.

[390] **Yoo SK, Huttenlocher A.** Spatiotemporal photolabeling of neutrophil trafficking during inflammation in live zebrafish. J Leukoc Biol. 2011;89(5):661-7.

[391] **Harun NO, Zou J, Zhang YA, Nie P, Secombes CJ.** The biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. Dev Comp Immunol. 2008;32(6):673-81.

[392] **Overland HS, Pettersen EF, Ronneseth A, Wergeland HI.** Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2010;28(1):193-204.

[393] **Mayumi M, Takeda Y, Hoshiko M, Serada K, Murata M, Moritomo T, et al.** Characterization of teleost phagocyte NADPH oxidase: molecular cloning and expression analysis of carp (*Cyprinus carpio*) phagocyte NADPH oxidase. Mol Immunol. 2008;45(6):1720-31.

[394] **Palic D, Andreasen CB, Menzel BW, Roth JA.** A rapid, direct assay to measure degranulation of primary granules in neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Fish Shellfish Immunol. 2005;19(3):217-27.

[395] **Palic D, Ostojic J, Andreasen CB, Roth JA.** Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol. 2007;31(8):805-16.

[396] **Palic D, Andreasen CB, Ostojic J, Tell RM, Roth JA.** Zebrafish (*Danio rerio*) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. J Immunol Methods. 2007;319(1-2):87-97.

[397] **Mantovani A, Cassatella MA, Costantini C, Jaillon S.** Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol. 2011;11(8):519-31.

[398] **Kumar V, Sharma A.** Neutrophils: Cinderella of innate immune system. Int Immunopharmacol. 2010;10(11):1325-34.

[399] **Panopoulos AD, Watowich SS.** Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. Cytokine. 2008;42(3):277-88.

[400] **Kanda N, Fukushige S, Murotsu T, Yoshida MC, Tsuchiya M, Asano S, et al.** Human gene coding for granulocyte-colony stimulating factor is assigned to the q21-q22 region of chromosome 17. Somat Cell Mol Genet. 1987;13(6):679-84.

[401] **Nicola NA, Metcalf D, Matsumoto M, Johnson GR.** Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. Identification as granulocyte colony-stimulating factor. J Biol Chem. 1983;258(14):9017-23.

[402] **Han SW, Ramesh N, Osborne WR.** Cloning and expression of the cDNA encoding rat granulocyte colony-stimulating factor. Gene. 1996;175(1-2):101-4.

[403] **Dunham SP, Onions DE.** Isolation, nucleotide sequence and expression of a cDNA encoding feline granulocyte colony-stimulating factor. Cytokine. 2001;14(6):347-51.

[404] **Heidari M, Kehrli ME, Jr.** Cloning, sequencing, and analysis of cDNA encoding bovine granulocyte-colony stimulating factor. Vet Immunol Immunopathol. 2000;73(2):183-91.

[405] **O'Brien PM, Seow HF, Rothel JS, Wood PR.** Cloning and sequencing of an ovine granulocyte colony-stimulating factor cDNA. DNA Seq. 1994;4(5):339-42.

[406] **Kulmburg P, Radke M, Mezes B, Mertelsmann R, Rosenthal FM.** Cloning and sequence analysis of the immediate promoter region and cDNA of porcine granulocyte colony-stimulating factor. Gene. 1997;197(1-2):361-5.

[407] **Leutz A, Beug H, Graf T.** Purification and characterization of cMGF, a novel chicken myelomonocytic growth factor. EMBO J. 1984;3(13):3191-7.

[408] **Leutz A, Damm K, Sterneck E, Kowenz E, Ness S, Frank R, et al.** Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor. EMBO J. 1989;8(1):175-81.

[409] **Santos MD, Yasuike M, Hirono I, Aoki T.** The granulocyte colony-stimulating factors (CSF3s) of fish and chicken. Immunogenetics. 2006;58(5-6):422-32.

[410] **Gibson MS, Kaiser P, Fife M.** Identification of chicken granulocyte colony-stimulating factor (G-CSF/CSF3): the previously described myelomonocytic growth factor is actually CSF3. J Interferon Cytokine Res. 2009;29(6):339-43.

[411] **Nam BH, An GH, Baeck GW, Kim MC, Kim JW, Park HJ, et al.** Molecular cloning and expression of cDNAs for two distinct granulocyte colony stimulating factor genes from black rockfish *Sebastes schlegelii*. Fish Shellfish Immunol. 2009;27(2):360-4.

[412] **Liongue C, Hall CJ, O'Connell BA, Crosier P, Ward AC.** Zebrafish granulocyte colony-stimulating factor receptor signaling promotes myelopoiesis and myeloid cell migration. Blood. 2009;113(11):2535-46.

[413] **Nagata S, Tsuchiya M, Asano S, Yamamoto O, Hirata Y, Kubota N, et al.** The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. EMBO J. 1986;5(3):575-81.

[414] **Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, et al.** Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature. 1986;319(6052):415-8.

[415] **Tsuchiya M, Kaziro Y, Nagata S.** The chromosomal gene structure for murine granulocyte colony-stimulating factor. Eur J Biochem. 1987;165(1):7-12.

[416] **Tsuchiya M, Asano S, Kaziro Y, Nagata S.** Isolation and characterization of the cDNA for murine granulocyte colony-stimulating factor. Proc Natl Acad Sci U S A. 1986;83(20):7633-7.

[417] **Watari K, Asano S, Shirafuji N, Kodo H, Ozawa K, Takaku F, et al.** Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. Blood. 1989;73(1):117-22.

[418] **Kawakami M, Tsutsumi H, Kumakawa T, Abe H, Hirai M, Kurosawa S, et al.** Levels of serum granulocyte colony-stimulating factor in patients with infections. Blood. 1990;76(10):1962-4.

[419] **Cheers C, Haigh AM, Kelso A, Metcalf D, Stanley ER, Young AM.** Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. Infect Immun. 1988;56(1):247-51.

[420] **Demetri GD, Griffin JD.** Granulocyte colony-stimulating factor and its receptor. Blood. 1991;78(11):2791-808.

[421] **Inazawa J, Fukunaga R, Seto Y, Nakagawa H, Misawa S, Abe T, et al.** Assignment of the human granulocyte colony-stimulating factor receptor gene (CSF3R) to chromosome 1 at region p35-p34.3. Genomics. 1991;10(4):1075-8.

[422] **Yoo J, Kappes SM, Stone RT, Beattie CW.** Linkage analysis assignment of bovine granulocyte colony stimulating factor receptor (G-CSFR) to chromosome 3. Mamm Genome. 1995;6(9):686-7.

[423] **Katzenback BA, Belosevic M.** Characterization of granulocyte colony stimulating factor receptor of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2012;36(1):199-207.

[424] **Seto Y, Fukunaga R, Nagata S.** Chromosomal gene organization of the human granulocyte colony-stimulating factor receptor. J Immunol. 1992;148(1):259-66.

[425] **Avalos BR.** Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood. 1996;88(3):761-77.

[426] **Shimoda K, Okamura S, Harada N, Niho Y.** Detection of the granulocyte colony-stimulating factor receptor using biotinylated granulocyte colony-stimulating factor: presence of granulocyte colony-stimulating factor receptor on CD34-positive hematopoietic progenitor cells. Res Exp Med (Berl). 1992;192(4):245-55.

[427] **Liongue C, Wright C, Russell AP, Ward AC.** Granulocyte colony-stimulating factor receptor: stimulating granulopoiesis and much more. Int J Biochem Cell Biol. 2009;41(12):2372-5.

[428] **Nicola NA, Metcalf D.** Binding of 125I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. J Cell Physiol. 1985;124(2):313-21.

[429] **Aritomi M, Kunishima N, Okamoto T, Kuroki R, Ota Y, Morikawa K.** Atomic structure of the GCSF-receptor complex showing a new cytokine-receptor recognition scheme. Nature. 1999;401(6754):713-7.

[430] **Tamada T, Honjo E, Maeda Y, Okamoto T, Ishibashi M, Tokunaga M, et al.** Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. Proc Natl Acad Sci U S A. 2006;103(9):3135-40.

[431] **Honjo E, Tamada T, Maeda Y, Koshiba T, Matsukura Y, Okamoto T, et al.** Crystallization of a 2:2 complex of granulocyte-colony stimulating factor (GCSF) with the ligand-binding region of the GCSF receptor. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2005;61(Pt 8):788-90.

[432] **Hiraoka O, Anaguchi H, Ota Y.** Formation of 1:1 complex of the cytokine receptor homologous region of granulocyte colony-stimulating factor receptor with ligand. Biosci Biotechnol Biochem. 1995;59(12):2351-4.

[433] **Hiraoka O, Anaguchi H, Ota Y.** Evidence for the ligand-induced conversion from a dimer to a tetramer of the granulocyte colony-stimulating factor receptor. FEBS Lett. 1994;356(2-3):255-60.

[434] **Richards MK, Liu F, Iwasaki H, Akashi K, Link DC.** Pivotal role of granulocyte colony-stimulating factor in the development of progenitors in the common myeloid pathway. Blood. 2003;102(10):3562-8.

[435] **Piper MG, Massullo PR, Loveland M, Druhan LJ, Kindwall-Keller TL, Ai J, et al.** Neutrophil elastase downmodulates native G-CSFR expression and granulocyte-macrophage colony formation. J Inflamm (Lond). 2010;7(1):5.

[436] **Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C, et al.** Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood. 1994;84(6):1737-46.

[437] **Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C.** Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of *Listeria monocytogenes*-infected mice. Blood. 1998;91(3):863-9.

[438] **Liu F, Wu HY, Wesselschmidt R, Kornaga T, Link DC.** Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. Immunity. 1996;5(5):491-501.

[439] **Hibbs ML, Quilici C, Kountouri N, Seymour JF, Armes JE, Burgess AW, et al.** Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an

inflammatory response in a sterile model of peritonitis. J Immunol. 2007;178(10):6435-43.

[440] **Liu F, Poursine-Laurent J, Wu HY, Link DC.** Interleukin-6 and the granulocyte colony-stimulating factor receptor are major independent regulators of granulopoiesis in vivo but are not required for lineage commitment or terminal differentiation. Blood. 1997;90(7):2583-90.

[441] **Seymour JF, Lieschke GJ, Grail D, Quilici C, Hodgson G, Dunn AR.** Mice lacking both granulocyte colony-stimulating factor (CSF) and granulocyte-macrophage CSF have impaired reproductive capacity, perturbed neonatal granulopoiesis, lung disease, amyloidosis, and reduced long-term survival. Blood. 1997;90(8):3037-49.

[442] **Hubel K, Dale DC, Liles WC.** Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma. J Infect Dis. 2002;185(10):1490-501.

[443] **Greenbaum AM, Link DC.** Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. Leukemia. 2011;25(2):211-7.

[444] **Tenen DG, Hromas R, Licht JD, Zhang DE.** Transcription factors, normal myeloid development, and leukemia. Blood. 1997;90(2):489-519.

[445] **Milbrandt J.** A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science. 1987;238(4828):797-9.

[446] **Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG, Tsai-Morris CH.** A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene Res. 1987;1(4):343-55.

[447] **Chavrier P, Zerial M, Lemaire P, Almendral J, Bravo R, Charnay P.** A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. EMBO J. 1988;7(1):29-35.

[448] **Patwardhan S, Gashler A, Siegel MG, Chang LC, Joseph LJ, Shows TB, et al.** EGR3, a novel member of the Egr family of genes encoding immediate-early transcription factors. Oncogene. 1991;6(6):917-28.

[449] **Crosby SD, Puetz JJ, Simburger KS, Fahrner TJ, Milbrandt J.** The early response gene NGFI-C encodes a zinc finger transcriptional activator and is a member of the GCGGGGGCG (GSG) element-binding protein family. Mol Cell Biol. 1991;11(8):3835-41.

[450] **Thiel G, Cibelli G.** Regulation of life and death by the zinc finger transcription factor Egr-1. J Cell Physiol. 2002;193(3):287-92.

[451] **Krishnaraju K, Nguyen HQ, Liebermann DA, Hoffman B.** The zinc finger transcription factor Egr-1 potentiates macrophage differentiation of hematopoietic cells. Mol Cell Biol. 1995;15(10):5499-507.

[452] **Krishnaraju K, Hoffman B, Liebermann DA.** Early growth response gene 1 stimulates development of hematopoietic progenitor cells along the

macrophage lineage at the expense of the granulocyte and erythroid lineages. Blood. 2001;97(5):1298-305.

[453] **Nguyen HQ, Hoffman-Liebermann B, Liebermann DA.** The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell. 1993;72(2):197-209.

[454] **Krishnaraju K, Hoffman B, Liebermann DA.** The zinc finger transcription factor Egr-1 activates macrophage differentiation in M1 myeloblastic leukemia cells. Blood. 1998;92(6):1957-66.

[455] **Carter JH, Tourtellotte WG.** Early growth response transcriptional regulators are dispensable for macrophage differentiation. J Immunol. 2007;178(5):3038-47.

[456] **Lee SL, Tourtellotte LC, Wesselschmidt RL, Milbrandt J.** Growth and differentiation proceeds normally in cells deficient in the immediate early gene NGFI-A. J Biol Chem. 1995;270(17):9971-7.

[457] **Gemelli C, Montanari M, Tenedini E, Zanocco Marani T, Vignudelli T, Siena M, et al.** Virally mediated MafB transduction induces the monocyte commitment of human $CD34^+$ hematopoietic stem/progenitor cells. Cell Death Differ. 2006;13(10):1686-96.

[458] **Drummond IA, Rohwer-Nutter P, Sukhatme VP.** The zebrafish egr1 gene encodes a highly conserved, zinc-finger transcriptional regulator. DNA Cell Biol. 1994;13(10):1047-55.

[459] **van der Meer LT, Jansen JH, van der Reijden BA.** Gfi1 and Gfi1b: key regulators of hematopoiesis. Leukemia. 2010;24(11):1834-43.

[460] **Karsunky H, Zeng H, Schmidt T, Zevnik B, Kluge R, Schmid KW, et al.** Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nat Genet. 2002;30(3):295-300.

[461] **Hock H, Hamblen MJ, Rooke HM, Traver D, Bronson RT, Cameron S, et al.** Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. Immunity. 2003;18(1):109-20.

[462] **Zarebski A, Velu CS, Baktula AM, Bourdeau T, Horman SR, Basu S, et al.** Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. Immunity. 2008;28(3):370-80.

[463] **de la Luz Sierra M, Sakakibara S, Gasperini P, Salvucci O, Jiang K, McCormick PJ, et al.** The transcription factor Gfi1 regulates G-CSF signaling and neutrophil development through the Ras activator RasGRP1. Blood. 2010;115(19):3970-9.

[464] **Dufourcq P, Rastegar S, Strahle U, Blader P.** Parapineal specific expression of gfi1 in the zebrafish epithalamus. Gene Expr Patterns. 2004;4(1):53-7.

[465] **Wei W, Wen L, Huang P, Zhang Z, Chen Y, Xiao A, et al.** Gfi1.1 regulates hematopoietic lineage differentiation during zebrafish embryogenesis. Cell Res. 2008;18(6):677-85.

[466] **Paun A, Pitha PM.** The IRF family, revisited. Biochimie. 2007;89(6-7):744-53.

[467] **Wang H, Morse HC, 3rd.** IRF8 regulates myeloid and B lymphoid lineage diversification. Immunol Res. 2009;43(1-3):109-17.

[468] **Holtschke T, Lohler J, Kanno Y, Fehr T, Giese N, Rosenbauer F, et al.** Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell. 1996;87(2):307-17.

[469] **Turcotte K, Gauthier S, Tuite A, Mullick A, Malo D, Gros P.** A mutation in the Icsbp1 gene causes susceptibility to infection and a chronic myeloid leukemia-like syndrome in BXH-2 mice. J Exp Med. 2005;201(6):881-90.

[470] **Tamura T, Nagamura-Inoue T, Shmeltzer Z, Kuwata T, Ozato K.** ICSBP directs bipotential myeloid progenitor cells to differentiate into mature macrophages. Immunity. 2000;13(2):155-65.

[471] **de Bruin AM, Libregts SF, Valkhof M, Boon L, Touw IP, Nolte MA.** Interferon-gamma induces monopoiesis and inhibits neutrophil development during inflammation. Blood. 2011.

[472] **Holland JW, Karim A, Wang T, Alnabulsi A, Scott J, Collet B, et al.** Molecular cloning and characterization of interferon regulatory factors 4 and 8 (IRF-4 and IRF-8) in rainbow trout, Oncorhynchus mykiss. Fish Shellfish Immunol. 2010;29(1):157-66.

[473] **Li L, Jin H, Xu J, Shi Y, Wen Z.** Irf8 regulates macrophage versus neutrophil fate during zebrafish primitive myelopoiesis. Blood. 2011;117(4):1359-69.

[474] **Eichmann A, Grapin-Botton A, Kelly L, Graf T, Le Douarin NM, Sieweke M.** The expression pattern of the mafB/kr gene in birds and mice reveals that the kreisler phenotype does not represent a null mutant. Mech Dev. 1997;65(1-2):111-22.

[475] **Kelly LM, Englmeier U, Lafon I, Sieweke MH, Graf T.** MafB is an inducer of monocytic differentiation. EMBO J. 2000;19(9):1987-97.

[476] **Aziz A, Soucie E, Sarrazin S, Sieweke MH.** MafB/c-Maf deficiency enables self-renewal of differentiated functional macrophages. Science. 2009;326(5954):867-71.

[477] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[478] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[479] **Barreda DR, Neumann NF, Belosevic M.** Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Dev Comp Immunol. 2000;24(4):395-406.

[480] **Barreda DR, Belosevic M.** Characterisation of growth enhancing factor production in different phases of in vitro fish macrophage development. Fish Shellfish Immunol. 2001;11(2):169-85.

[481] **Hanington PC, Barreda DR, Belosevic M.** A novel hematopoietic granulin induces proliferation of goldfish (*Carassius auratus* L.) macrophages. J Biol Chem. 2006;281(15):9963-70.

Chapter 3: Materials and Methods

3.1 Fish

Goldfish (*Carassius auratus* L.), approximately 10-15 centimeters, were obtained from Aquatic Imports (Calgary, AB). Fish were maintained in tanks with a continuous flow water system at 20^{O}C and with a 14 hr light/10 hr dark period in the aquatic facilities in the Biological Sciences building at the University of Alberta. Fish were fed until satiated daily and were acclimated for at least three weeks prior to use in the experiments. During this time period, fish were monitored for any signs of disease to ensure they were healthy. Prior to handling, fish were sedated using tricaine methane sulfonate (TMS) solution of 40-50 mg/L of water. When necessary, individual fish were marked by fin clipping. The fish in the aquatic facility were maintained and manipulated according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada).

3.2 Fish serum

3.2.1 Carp serum

Carp (*Cyprinus carpio*) were bled from the caudal vein every 4-6 weeks using a 21-gauge needle attached to a 3 mL syringe. Blood was pooled and allowed to clot overnight at 4^{0}C. The next day, blood was centrifuged for 30 minutes at 1000 x *g*. Serum was collected, heat inactivated at 56^{0}C for 30 minutes, sterilized by filtration using a 0.22 μm filter, and frozen at -20^{O}C until use in the experiments. Carp serum was used for the cultivation of goldfish primary kidney macrophage (PKM) cultures and neutrophil cultures.

3.2.2 Goldfish serum

Goldfish serum was collected and prepared using the same procedures as those for carp serum and was used for cultivation of *Trypanosoma carassii*.

3.3 Fish primary cell culture

3.3.1 Culture medium

The culture medium used for cultivation of goldfish primary kidney macrophages and primary kidney neutrophils, NMGFL-15, was previously described in [1, 2]. The composition of incomplete NMGFL-15 medium is shown in Table 3.1. The composition of the nucleic acid precursor solution is shown in Table 3.2, and that of Hank's Balanced Salt Solution (HBSS) in Table 3.3. Complete NMGFL-15 medium contained 10% heat-inactivated newborn calf serum, 5% heat-inactivated carp serum, 100 U/mL penicillin/100 μg/mL streptomycin (P/S) and 100 μg/mL gentamycin.

3.3.2 Isolation of goldfish leukocytes

3.3.2.1 Primary kidney macrophage (PKM) cultures

Goldfish were anesthetized with TMS and their spines severed using scissors. The isolation and cultivation of goldfish primary kidney macrophages from the kidney was performed as previously described [2, 3]. Briefly, the entire body kidney tissue was removed and placed into a Petri dish containing 10 mL of cold incomplete medium (without serum). The contents of the Petri dish was

transferred to a sterile laminar flow hood and the kidney tissue was gently homogenized by passing the tissue through a wire mesh screen using a sterile plunger from a 3 mL syringe. Cells were washed through the mesh screen using incomplete NMGLF-15 medium containing heparin and P/S to achieve a single cell suspension. The resulting cell suspension was allowed to sit for 5 minutes to allow debris to settle, and then layered over a 51% Percoll solution (51 mL Percoll, 10 mL 10 x HBSS, 39 mL NMGFL-15 medium) and centrifuged for 25 minutes at 400 x *g*. Cells at the 51% Percoll/medium interface were transferred to a new sterile 15 mL tube containing 10 mL of incomplete NMGFL-15 medium. Cells were centrifuged for 10 minutes at 230 x *g*. This wash step was repeated before re-suspending the isolated kidney leukocytes in complete NMGFL-15 medium. Leukocytes were enumerated using a haemocytometer and cell viability assessed using Trypan blue exclusion method. The cell viability was always greater than 95%.

3.3.2.2 Primary kidney neutrophils

The isolation of goldfish kidney leukocytes was performed as previously described above and in ``[2, 3] with the following modifications. Briefly, the kidney cell suspension was layered on 51% Percoll, centrifuged at 400 x *g* for 25 minutes and the cells at the 51% Percoll/medium interface removed. All remaining Percoll was removed, leaving behind the red blood cell/neutrophil pellet found at the bottom of the tube. The red blood cells were lysed using an ice-cold 1X red blood cell lysis buffer (144mM NH_4Cl, 17mM Tris, pH = 7.2). Approximately 3-5 mLs of lysis buffer were added to the pellet and allowed to sit

for approximately 10-15 minutes on ice. Tubes were centrifuged for 5 minutes at 230 x *g*. Following centrifugation, the lysis buffer was removed and one mL of incomplete medium added. The layer of membranes found on top of the neutrophil cell layer was carefully removed using a pipette. The contaminating monocytes/macrophages were removed by allowing the monocytes/macrophages to adhere to the bottom of culture vessels, and non-adherent neutrophils harvested (see section 1.3.3.2 below).

3.3.3 Cell cultivation

3.3.3.1 Primary kidney macrophages

Kidney leukocytes were seeded at a density of ~ 1x 10^6 cells/mL in complete NMGFL-15 medium and cultured at 20^{o}C in the absence of added CO_2. These primary kidney macrophage (PKM) cultures were composed of three distinct cell populations consisting of early progenitors (R1), monocytes (R3), and mature macrophages (R2). Primary kidney macrophage cultures are a heterogeneous population of cells and have been extensively characterized by flow cytometry, morphology, cytochemistry and function [2, 3]. Cell conditioned medium (CCM) from PKM cultures was collected from day 6-8 of cultivation. In some cases, subsequent PKM cultures were supplemented with 25% (v/v) CCM.

In some experiments, kidney leukocytes from four individual fish (n = 4) were seeded at 2 mL/well in a 6-well culture plate at a concentration of 1 x10^6 cells/mL in complete NMGFL-15 medium. Cells were harvested at 0, 2, 4 and 8 days post culture using a cell scraper. Cell suspensions were centrifuged at 300 x *g* for 5 min to pellet cells prior to RNA isolation using Trizol.

3.3.3.2 Primary kidney neutrophils

In some experiments, cells from 5-6 fish were pooled and washed twice in incomplete medium, centrifuged at 400 x *g* for 10 minutes, re-suspended in 15 mL of complete medium and seeded into plug-seal 75 cm^3 polystyrene cell culture flasks containing complete medium overnight prior to use. In other experiments, cells from individual fish were kept separate and cultured overnight in plug-seal 25 cm^3 flasks in 5-6 mL of complete NMGFL-15 medium. Isolated neutrophils were non-adherent and, based on this property, were cultured overnight at 20°C in the absence of added CO_2 to aid in the removal of any contaminating monocytes/macrophages as these cells are known for their adherence. Neutrophils remained in suspension.

3.4 Isolation of cell populations from goldfish tissues

3.4.1 Peripheral blood leukocytes and splenocytes

To isolate peripheral blood leukocytes, fish were bled from the caudal vein with a heparin-coated syringe to prevent clotting. Blood was diluted 1:4 with incomplete NMGFL-15 containing 1% P/S and heparin. Splenocytes were obtained by gently homogenizing the spleen using a wire mesh with addition of NMGFL-15 medium containing heparin and P/S. Debris was allowed to settle out before layering the spleen cell suspension on 51% Percoll. Mixtures of peripheral blood leukocytes or splenocytes were layered over 51% Percoll, and centrifuging at 430 x *g* for 25 minutes. The buffy coat and the leukocytes found in the red blood cell pellet (following RBC lysis) were combined and washed twice with incomplete NMGFL-15 prior to RNA isolation using Trizol (Invitrogen).

3.4.2 Mononuclear cells from tissues

The kidney, spleen, liver, and brain tissues were isolated from each fish and homogenized in incomplete NMGFL-15 medium containing heparin and 2% P/S by gently pushing them through wire mesh screens. The resulting single cell suspension was allowed to settle for 5 minutes to remove debris, and then layered over 51% Percoll. Cells were centrifuged at 300 x *g* for 25 minutes, and the buffy layer removed and washed twice in incomplete NMGFL-15 medium with centrifugation at 230 x *g* for 10 minutes.

3.4.3 Neutrophils from peripheral blood and spleen tissue

To isolate neutrophils from the peripheral blood, fish were bled from the caudal vein using a 1 mL syringe fitted with a 25-gauge needle containing 50 μL of heparin to prevent clotting. Blood was diluted 1:4 in incomplete NMGFL-15 medium. To isolate neutrophils from the spleen, spleen tissue was harvested and pushed through a wire mesh screen using a sterile plunger from a 3 mL syringe. Cells were rinsed from the wire mesh screen using incomplete NMGFL-15 medium containing P/S and heparin. The resulting single cell suspensions from the blood or spleen were layered over a discontinuous Percoll gradient consisting of 51%, 60% and 75% Percoll layers. Each 2 mL layer was under-layered with the denser Percoll mixture using a 3 mL syringe fitted with an 18 gauge needle. Cells were centrifuged at 400 x *g* for 25 minutes. Cell layers at each of the Percoll interfaces were collected and transferred to another sterile tube. Cells were washed with 5 mL of incomplete NMGFL-15 medium to remove traces of

Percoll. Residual red blood cells were lysed with 1X RBC lysis buffer, centrifuged at 230 x *g* for 5 minutes, membranes removed and re-suspended in incomplete NMGFL-15 medium.

3.5 Cell sorting

3.5.1 Sorting of goldfish R1 progenitor cells from PKM cultures

Freshly isolated cells from goldfish kidney, or cells from day 1, 2, 3 or 6 of PKM cultivation were centrifuged at 230 x *g* for 10 minutes. Cells were re-suspended in fresh complete NMGFL-15 medium and viable cells enumerated using a haemocytometer and Trypan blue. The cells were adjusted to a concentration of ~ 5-10 x 10^6 cells/mL in complete NMGFL-15 medium containing 2% P/S for cell sorting using a FACS Aria flow cytometer at the Department of Medical Microbiology and Immunology, University of Alberta, flow cytometry facility. Cells were sorted into tubes containing 7 mL of complete NMGFL-15 medium containing 2% P/S. Following sorting, cells were centrifuged at 230 x *g* for 15 minutes to collect the sorted cells.

3.5.2 Sorting of goldfish R3 monocytes and R2 macrophages

PKMs were cultured for 3-4 days for the isolation of monocytes, or 6-8 days for the isolation of macrophages. Following the cultivation time indicated, PKMs were harvested, centrifuged at 230 x *g* for 10 minutes, and adjusted to a concentration of ~ 2 x 10^6 cells/mL. Monocytes and macrophages were sorted using a Becton/Dickinson FACS Calibur flow cytometer based on their size and internal complexity of the cells. Cells were sorted into 50 mL centrifuge tubes

coated with newborn calf serum (NCS) and tubes contained 3-5 mLs of NCS with 5% P/S. Tubes containing sorted cells were centrifuged at 230 x *g* for 10 minutes to collect the sorted cells.

3.6 Fish cell lines

3.6.1 CCL71 cells

Goldfish CCL71 fin fibroblast cells were obtained from ATCC. CCL71 cells were cultured in minimal essential medium (Eagle) and Hanks' balanced salt solution containing 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 4.17 mM sodium bicarbonate, and 10% fetal bovine serum (FBS). Cells were grown at 20°C without added CO_2. Medium was replaced bi-weekly. Approximately every 4-5 days, confluent cells were rinsed with PBS-A and treated with 0.25% trypsin-0.03% EDTA to detach adherent cells. Trypsin was inactivated by adding complete MEM. Cells were sub-cultivated at a 1:4 dilution as recommended by the ATCC instructions.

3.6.2 Catfish 3B11 B-cell line

Catfish 3B11 B-cells were a kind gift from Dr. James Stafford, University of Alberta. Cells were maintained in AL3 medium (50% AIM V medium, 50% L-15 medium, 0.5% P/S, 25 mg/L Gentamicin, 11.9 mM sodium bicarbonate, 50 mM beta-mercaptoethanol, 3% heat inactivated catfish serum) at 27°C, 5% CO_2. These cells grow in suspension, and do not require trypsin treatment. Cells were passed every 4 days at a 1:40 dilution.

3.7 Mammalian cell lines

3.7.1 HEK293T and CHO-Pro5 cells

Cells were maintained in DMEM supplemented with 10% FBS, 1% P/S, 1% non-essential amino acids, 10 mM HEPES and 44 mM sodium bicarbonate at 37°C with 5% CO_2. Approximately every 4-5 days, when cells became confluent, cells were rinsed with PBS-A, and treated with 0.25% trypsin/ 0.03% EDTA to detach cells. Trypsin was neutralized by the addition of complete medium. Cells were passed at a 1:10 dilution.

3.7.2 RBL2H3 cell line

The rat basophil RBL2H3 cell line was grown at 37°C with 5% CO_2 in minimal essential medium with Earle's balanced salt solution supplemented with 2 mM L-glutamine, 1% P/S (Gibco), and 10% heat-inactivated FBS as described in [4]. Cells were passed every 4 days by harvesting cells in an RBL harvest buffer (1.5 mM EDTA, 135 mM NaCl, 20 mM HEPES, 5 mM KCl, pH 7.4) at 37°C with 5% CO_2 for 10 minutes, followed by gentle pipetting to remove cells. Cells were seeded into a new flask at a sub-cultivation ratio of 1:10.

3.8 Pathogens

3.8.1 *Aeromonas salmonicida* A449

3.8.1.1 Determination of Aeromonas salmonicida A449 growth curve

To determine the growth kinetics of *Aeromonas salmonicida* A449, colonies were grown for 48-72 hours at 18°C on Tryptic Soy Agar + 20 μg/mL

chloramphenicol (Sigma). A single colony was chosen and used to inoculate 10 mL of Tryptic Soy Broth + 20 μg/mL chloramphenicol and incubated for 24 hours at 18°C with shaking at 250 rpm. A 1:100 dilution of the 24 hour culture was added to 200 mL of Tryptic Soy Broth + 20 μg/mL chloramphenicol and incubated at 18°C with shaking at 250 rpm. A 1 mL sample of the culture was taken every one to two hours and used to measure the optical density (Fig. 3.1A) and for enumeration of colony forming units (Fig. 3.2B).

3.8.1.2 Live Aeromonas salmonicida A449

Aeromonas salmonicida A449 was a kind gift from Dr. Jessica Boyd (NRC Institute, Halifax, Canada). This strain is virulent, possesses an A layer and is aggregating. Glycerol stocks of *A. salmonicida* A449 stored at -80°C were used to streak Tryptic Soy Agar (TSA) + 20 μg/mL chloramphenicol (Sigma) plates and incubated at 18°C for 72 hours. Colonies were stored at 4°C on TSA + 20 μg/mL chloramphenicol (Sigma) and a single colony was used to inoculate 5 mLs of tryptic soy broth (TSB) + 20 μg/mL chloramphenicol that was grown for 24 hours at 18°C with shaking to stationary phase. A 1:100 dilution of the 24 hour culture was used to inoculate 100 mL of TSB + chloramphenicol and cultured until mid-log phase at 18°C with shaking. Cultures were harvested and washed twice with either HBSS with Ca^{2+} and Mg^{2+} without phenol red for degranulation assays, or in incomplete medium for use in respiratory burst and chemotaxis assays. Due to the aggregating nature of the bacteria a small sample was diluted one-to-one in 2% SDS which mitigated the clumping and allowed the enumeration of the bacteria using a haemocytometer and a light microscope.

3.8.1.3 Heat-killed Aeromonas salmonicida A449

Colonies were stored at 4°C on TSA + 20 μg/mL chloramphenicol (Sigma) and a single colony was used to inoculate 5 mL of TSB + 20 μg/mL chloramphenicol that was grown to stationary phase for 24 hrs at 18°C with shaking. Bacteria were washed twice and re-suspended in sterile 1x PBS, pH 7.4 to the original volume of the culture. Prior to heat-killing, a sub-sample of *A. salmonicida* culture was withdrawn and used for serial dilutions in TSB containing 1% SDS prior to plating on duplicate TSA + chloramphenicol plates to mitigate clumping and allow for the enumeration of individual colony forming units (CFUs). Plates were grown at 18°C for 72 hours prior to enumerating colonies. *A. salmonicida* was then heat-killed for 45 min at 60°C in a circulating water bath. Following incubation, a sample of the bacteria was plated on TSB + chloramphenicol plates to ensure bacteria were non-viable. Heat-killed *A. salmonicida* A449 was stored at -20°C until used.

3.8.1.4 Generation of A. salmonicida A449 conditioned medium

The conditioned supernatants were generated by incubating 1 x 10^9 bacterial cells/mL in incomplete NMGFL-15 medium at 20°C for 1 hour. The bacteria were removed by centrifugation at 2,200 x *g* for 5 minutes and the supernatant filter sterilized using a 0.22 μm filter (Millipore). Conditioned supernatants were generated for use in the neutrophil chemotaxis assay.

3.8.2 *Trypanosoma carassii* (syn *T. danilewskyi*)

Trypanosoma carassii (strain TrCa) (syn. with *T. danilewskyi*) was isolated from a crucian carp (*C. carassius*) by Dr. J. Lom in 1977. The parasites were obtained from Dr. P.T.K. Woo, University of Guelph, Ontario, Canada. Trypanosomes were maintained *in vitro* in our laboratory as glycerol stocks at -80°C as well as serial passages from a 6-7 day old stock culture in TDL-15 medium containing 10% heat-inactivated goldfish serum. The recipe for TDL-15 medium is shown in Table 3.4. Trypanosomes used for all assays were cultured *in vitro* and used from 6-7 day old stock cultures as previously described [5].

3.9 Cytochemical staining

3.9.1 Hematoxylin and Eosin

Suspension cells from overnight neutrophil cultures were washed twice with incomplete NMGFL-15 medium. Viable cells were enumerated using a haemocytometer and Trypan blue and re-suspended to a cell concentration of 1 x 10^6 cells/mL. One-hundred microlitres of the neutrophil cell suspension was spun onto pre-cleaned glass slides at 55 x *g* for 7 minutes using a cytospin (Shandon Instruments). Cells were fixed by incubation in 70% methanol for 1 minute. Cells were rinsed in distilled water, stained with hematoxylin for a minute, rinsed three times with distilled water, and counter-stained with eosin for 1 minute followed by rinsing with distilled water. Slides were allowed to air-dry prior observation using bright field microscopy.

3.9.2 Sudan Black

For Sudan Black staining (Sigma) 1 x 10^5 cells were spun onto a glass slide at 55 x *g* for 7 minutes using a cytospin (Shandon Instruments). Cells were fixed with 75% gluteraldehyde: 25% acetone fixative solution for one minute at 4ºC, rinsed with distilled water, and slides immersed in Sudan Black staining reagent for five minutes with gentle agitation. Slides were thoroughly rinsed with 70% ethanol to remove excess Sudan Black staining and further rinsed in distilled water. Cells were counterstained with Gill's 3 solution for five minutes and rinsed with tap water for two minutes. Slides were air-dried prior to observation using bright field microscopy.

3.9.3 α-napthyl acetate esterase

For α-naphthyl acetate esterase staining (Sigma), 1 x 10^5 neutrophils were fixed to the slide with a 3.1: 8.1: 1 ratio of citrate-acetone- 37% formaldehyde (CAF) solution for one minute with gentle agitation at room temperature. The α-naphthyl acetate esterase staining solution was prepared according to the manufacturer's recipe and transferred to Coplin jars and placed in a water bath at 37ºC. The temperature of the solution was checked with a thermometer to ensure the solution was at temperature before addition of the slides. Cells were stained with the staining solution for 30 minutes at 37ºC. Cells were counterstained with Gill's 3 Hematoxylin solution for 2 minutes at room temperature. Slides were washed with distilled water between each step, except for the final wash after Gill's solution that was with tap water.

3.9.4 Acid phosphatase

For acid phosphatase staining (Sigma), 1 x 10^5 neutrophil cells were fixed to a glass slide with a 3.1: 8.1: 1 ratio of citrate-acetone- 37% formaldehyde (CAF) solution for 1 minute at room temperature with gentle agitation. The acid phosphatase solution was prepared with or without tartrate and transferred to individual Coplin jars. Jars and solutions were pre-warmed to 37°C in a water bath, prior to addition of slides. Slides were placed in the Coplin jars and incubated in acid phosphatase solution with or without tartrate for 1 hour at 37°C. Cells were counterstained with Gill's 3 Hematoxylin solution for 2 minutes at room temperature, followed by rinsing with tap water. All other rinses between stains were done using distilled water.

3.9.5 Myeloperoxidase

Neutrophils, 1 x 10^5 cells, were cytospun at 55 x *g* for 7 minutes onto glass slides. The myeloperoxidase staining protocol was followed according to the manufacturer's instructions (Sigma). Briefly, cells were fixed with at 75% gluteraldehyde : 25% acetone fixative solutions for one minute at 4°C. Slides were rinsed briefly with distilled water before incubation for 45 seconds in the Diaminobenzidine/Peroxide solution. Slides were then added to a Coplin jar containing Copper Nitrate solution for 2 minutes with gentle agitation, dipped in Hematoxylin Solution Gill no. 3 four times, and dipped in Scott's Tap Water Substitute Working Solution 6 times. Between each solution, slides were rinsed with distilled water.

3.9.6 Periodic Acid Schiff

Neutrophils, 1 x 10^5 cells in 100 μL, were cytospun onto glass slides at 55 x *g* for 7 minutes. Fresh fixative solution consisting of 10% formaldehyde : 90% ethanol (95%) was used to fix neutrophils to glass slides. The protocol for staining cells was followed according to the manufacturer's directions (Sigma). Briefly, slides were immersed in Periodic Acid Solution (PAS) for five minutes, followed by counterstaining with Hematoxyline Solution, Gill No. 3 for 1.5 minutes. Between fixative and staining steps, slides were rinsed with distilled water. Following Hematoxylin staining, slides were rinsed under tap water. All steps were performed at room temperature.

3.9.7 Visualization of cytochemical stained cells

One to three days following cell staining, photomicrographs were generated using Leica DMR light microscope using a bright field 100X objective (1000X magnification) under oil immersion.

3.10 Identification, sequencing and *in silico* analysis of goldfish cell surface receptors, ligands and transcription factors.

3.10.1 Primers

Primers used in this thesis for vector specific sequencing are shown in Table 3.5. The primers used in homology based PCR, RACE-PCR, RT-PCR and primers used to generate a probe for Southern blotting are shown in Table 3.6.

3.10.2 RNA isolation

3.10.2.1 Trizol method

RNA was isolated from goldfish tissues or cells (> 1 x 10^6) using Trizol (Invitrogen) according to the manufacturer's instructions. Briefly, tissue or cells were place in an RNAse free Eppendorf tube, lysed in 1 mL of Trizol reagent and homogenized by continually filling and expelling the tissue or cell through a 1 mL syringe fitted with a 18G, 21G and finally, a 25G needle. Following homogenization, the Trizol mixture was allowed to sit at room temperature for 5 minutes prior to the addition of 200 μL of chloroform. Tubes were vigorously shaken, allowed to settle for 2-3 minutes, and centrifuged at 10,800 x *g* for 15 minutes in a refrigerated microcentrifuge at 4^{o}C. The aqueous (clear) layer was extracted and transferred to a new 1.5 mL Eppendorf tube containing 200 μL of chloroform. The process of mixing and centrifuging was repeated. The double chloroform extraction was used to remove traces of phenols. The aqueous layer was transferred to a new 1.5 mL Eppendorf tube containing 500 μL of isopropanol. Tubes were inverted 15 times. At this point, when the RNA was being collected from low numbers of cells, a 1:20 dilution of 3 M sodium acetate was added to the tubes and the samples placed at -20^{o}C overnight. Following addition of isopropanol, samples were centrifuged at 10,800 x *g* for 10 minutes to pellet the RNA. Supernatants were aspirated, and the RNA pellet washed twice with 1 mL of 75% reagent grade ethanol followed by centrifugation at 8,500 x *g* for 5 minutes. Ethanol was aspirated and pellets allowed to air-dry for 5-10 minutes. Nuclease-free water was used to re-suspend the RNA pellet. RNA samples were treated with DNAseI to remove contaminating genomic DNA, and

the DNAseI enzyme inactivated by incubating the sample at 65ºC for 5 minutes. The nucleic acid concentration was quantified using a Nanodrop apparatus at an absorbance of 260 nm. Samples were also read at absorbences of 230 nm and 280 nm to determine phenolic and protein contamination.

3.10.2.2 MicroRNA spin column method

MicroRNA spin columns (ZymoResearch) were used for RNA isolation from progenitor cells ranging in concentrations from 2 x 10^5 to 1 x 10^6 cells. Cells were re-suspended in 400 μL of RNA lysis buffer and the sample homogenized using a 1 mL syringe fitted with a 25G needle. The samples were centrifuged for 1 minute at 12,000 x *g*, and the supernatant transferred to the IIC column placed in a collection tube. The IIC column was centrifuged for 30 seconds at 8,000 x *g*, and to the flow through in the collection tube, 320 μL of 100% reagent grade ethanol was added and mixed by pipetting. The mixture was transferred to an IC column in a collection tube and centrifuged for 1 minute at 12,000 x *g*. At this point, RNA was treated with a 20 μL of DNAse I enzyme solution for 20 minutes at 37ºC to remove residual traces of genomic DNA. The column was centrifuged at 12,000 x *g* to remove the DNAseI enzyme and buffer, and the column subsequently washed with 400 μL of RNA prep buffer, followed by 800 μL and 400 μl of RNA wash buffer. The column was centrifuged at 12,000 x *g* for 1 minute between steps, and for 2 minutes after the last step to remove all traces of ethanol. The IC columns were then transferred to a clean 1.5 mL Eppendorf tube and 12 μL of nuclease free water added to the filter of the IC column. Columns stood for 2 minutes prior to centrifugation at 10,000 x *g* for 3 seconds to

collect the RNA sample. Nucleic acid concentrations were determined using a Nanodrop, as described above.

3.10.3 cDNA synthesis

cDNA synthesis from RNA was performed using the Superscript II cDNA synthesis kit (Invitrogen) or the Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's specifications. RNA levels were quantified (as described in section 1.9.2) and normalized prior to cDNA synthesis. In all cDNA synthesis reactions, Oligo dT was used as the primer.

3.10.4 RT-PCR

Target mRNA transcripts were amplified by adding 2 μL of cDNA template to 81 μL of nuclease free water, 10 μL of 10X PCR buffer (1 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% w/v gelatin), 2 μL of 20 mM primer solution, 2 μL of 10 mM dNTPs, and 1 μL of Taq polymerase. Reactions were amplified in an Eppendorf Mastercycler Gradient thermocycler. The general thermocycling program consisted of an initial denaturation step of 94^{o}C for 2 minutes, followed by 25-30 cycles of 94^{o}C for 30 seconds; 55^{o}C ± 10^{o}C for 30 seconds; 72^{o}C for 2 minutes, and a final extension of 72^{o}C for 10 minutes.

For colony PCR, the PCR mixture was set up in a similar manner as described above, however, instead of cDNA template, a single colony was picked and swirled into 12.5 μL of reaction mixture. The thermocycling program for M13 forward and reverse primers is as follows: an initial denaturation step of 94^{o}C for 10 minutes, followed by 25 cycles of 94^{o}C for 30 seconds; 50^{o}C for 20 seconds;

72°C for 2 minutes, and a final extension of 72°C for 2 minutes. For T7 forward and BGH reverse primers, the annealing temperature was set to 55°C. The extension time of 2 minutes was varied based on the expected size of the insert, and was generally set at 1 minute per 1000 bp. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

3.10.5 Cloning into pCR2.1 TOPO TA vector

Bands of interest were excised from gels and purified using the Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. All bands were eluted in 30 μL of elution buffer provided with the kit. Two microlitres of the purified PCR product was transferred to a 0.6 mL tube on ice containing 0.5 μL of the salt solution provided. To this mixture, 0.5 μL of pCR2.1 TOPO TA vector was added and gently mixed by swirling the pipette tip in the mixture. The tube was then placed at room temperature for 5-10 minutes. For larger inserts (> 1500 bp), the incubation time was extended to 20-30 minutes. During the final five minutes of incubation, One Shot Top 10 *E. coli* competent cells, stored frozen at -80°C, were placed on ice and allowed to thaw. The entire 3 μL PCR product/salt/vector mixture was added transferred to the competent cells, mixed by gently swirling the pipette tip in the solution, and incubated for 5 minutes on ice. Cells were heat-shocked at 42°C for 30 seconds and allowed to recover for 1-2 minutes on ice. Two hundred microlitres of SOC medium, provided by the manufacturer, was added, and cells incubated at 37°C for 45 minutes with shaking at 250 rpm. Cells were plated on LB agar plates containing 100 μg/mL ampicillin and incubated

overnight at 37ºC. The next day, the presence of inserts was assessed using EcoRI restriction digestion or by colony PCR.

3.10.6 DNA sequencing and *in silico* analysis

3.10.6.1 General approach

Generated amplicons were gel purified using the QIA Gel Extraction kit (Qiagen) and cloned into the TOPO TA pCR2.1 vector (Invitrogen). Positive colonies were identified by colony PCR using the vector specific M13 forward and reverse primers, isolated using the QIAspin Miniprep kit (Qiagen) and sequenced using an ET terminator cycle sequencing dye and a PE Applied Biosystems 377 automated sequencer. Single pass sequences were analyzed using 4peaks software (http://mekentosj.com/4peaks/) and sequences aligned and analyzed using BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.10.6.2 DNA sequencing and in silico analyses of goldfish kita and kitla

The sequences for goldfish *kita* and *kitla* were identified using homology based PCR using primers (IDT) designed against zebrafish *kita* and *kitla* sequences in the NCBI database, accession numbers NM131053 and AY929069, respectively. From this initial fragment, RACE PCR (BD Sciences, Clonetech) was performed to obtain a partial open reading frame for goldfish *kita* and the full open reading frame for *kitla* according to manufacturer's specifications.

Protein sequences, conserved motifs, secretion signals, trans-membrane domains and potential O- and N-glycosylation sites were predicted using programs from the ExPASy website (http://ca.expasy.org/) and the conserved domains program on NCBI (http://www.ncbi.nlm.nih.gov/). The goldfish *kita* (FJ907418) and *kitla* (FJ907419) mRNA sequences have been submitted to GenBank.

3.10.6.3 DNA sequencing and in silico analyses of goldfish gcsfr

The sequence for goldfish GCSFR was identified using homology based PCR using primers (IDT) designed against zebrafish GCSFR sequence in the NCBI database (Accession number NM_001113377). From this initial fragment, RACE PCR (BD Sciences, Clonetech) was performed to obtain a full open reading frame for goldfish GCSFR according to manufacturer's specifications

The predicted protein sequences, conserved motifs, a secretion signal cleavage site, transmembrane domains and cytoplasmic domain were predicted using programs from the ExPASy website (http://ca.expasy.org/). The goldfish GCSFR sequence (JF922012) has been submitted to GenBank.

3.10.6.4 Identification, cloning and sequencing of goldfish transcription factors

Primers were designed (IDT) using homology based PCR. Genbank accession numbers for the sequences used for homology based PCR: Runx1 AF391125, cMyb NM_131266, GATA2 AB429308, Egr1 NM_131248, CEBPα NM_131885, GATA1 BC164788, Lmo2 NM_131111, GATA3 AB302069, Pax5

AB429310. Partial sequences for goldfish PU.1, cJun, and MafB were obtained from a differential cross screen conducted in our lab previously [6]. Quantitative PCR primers were designed using the 7500 fast primer design software based on the full or partial sequences of the goldfish transcription factors obtained.

3.11 Southern blotting

3.11.1 Isolation of genomic DNA from blood

Genomic DNA was isolated from goldfish whole blood using the FlexiGene DNA kit (Qiagen) according to manufacturer's instructions with the following modifications. Briefly, goldfish were bled using a needle and syringe containing 5 mM EDTA as an anticoagulant. For every 0.5 mL of blood, 2 mLs of buffer FG1 was added followed immediately by inverting the tube 5 times. The mixture was centrifuged for 5 minutes at 2000 x *g* to pellet nuclei. Supernatants were aspirated and 3 mLs of buffer FG2 was added to the pellet followed immediately by mixing. The re-suspended solution was incubated for 10 minutes at 65^{o}C. DNA was then precipitated using isopropanol, and washed with 70% ethanol before being dissolved in 500 μL of Buffer FG3.

3.11.2 Restriction digestion of genomic DNA

Isolated DNA was quantified and 10 μg of DNA was digested with the restriction enzymes KpnI, HindIII, XbaI, PstI, PaeI, or TaiI overnight. Digestions were set up in 100 μL reactions, containing 50 U of enzyme. The following morning, 50 U of fresh enzyme was added, and the digestion done for an additional 2 hrs. Following digestion, DNA was precipitated using 1:10 ratio of 3

M sodium acetate and a 1:1 volume of isopropyl alcohol and concentrated to 20 μL.

3.11.3 Electrophoresis and transfer of digested genomic DNA

The entire digested DNA sample (10 μg) was run on a 0.8% agarose gel at 80 V for 11 hours. The gel was then depurinated with 0.2 M HCl for 10 minutes, denatured using 1.5 M NaCl, 0.5 M NaOH for 30 minutes and neutralized with 3 M NaCl, 0.5 M Tris HCl, pH 7 for 30 minutes. The buffers used in the denaturation and neutralization steps were replaced with 100 mL of fresh buffer after 15 minutes. All steps were performed with rocking. Nucleic acid from the gel was transferred to a Hybond N+ nylon membrane (Amersham) using 20x SSC (3 M NaCl, 0.3 M sodium citrate) and capillary action overnight [7]. Following transfer, the DNA was UV-cross-linked to the membrane.

3.11.4 Labeling and detection of bound probe

The Gene Images AlkPhos Direct Labeling and Detection System (Amersham) was used to probe the membrane according to manufacturer's specifications. Hybridization and post-hybridization stringency washes were performed at 60^{o}C in a hybridization oven with an integral rotisserie device. The *GCSFR* probe corresponded to the predicted exon 2, which encodes for the Ig-like domain, and a probe concentration of 10 ng/mL was used for the hybridization step. Following washing steps, bound probe was detected using chemilluminescent reagents provided with the kit. Film was exposed to the nylon membrane for 1 or 2 hours and developed.

3.12 Quantitative PCR

3.12.1 Primers

All qPCR primers used in this thesis were designed with the Primer Express software (Applied Biosystems) and are shown in Table 3.7. Primers for qPCR were validated by running primers with 1:2 serial dilutions of cDNA and creating a standard curve, used in determining in the R^2 value, y-intercept, and efficiency of the primer set using the 7500 Fast software. All primer sets were chosen with an R^2 value of 0.997 or higher, a y-intercept value of -3.0 to -3.2, and an efficiency of 85% or higher. Melt curves were analyzed to ensure a single melting peak, and qPCR products were run on a gel, excised, and sequenced to ensure the correct amplicon was being amplified.

3.12.2 Quantitative PCR cycling conditions and analysis

All quantitative expressions of goldfish genes were performed using SYBR green reagents and an Applied Biosciences 7500 Fast Real Time Machine and elongation factor 1 alpha was employed as an endogenous control. Thermocycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A melting curve step was added to the end of this protocol. Data were analyzed using the 7500 fast software (Applied Biosciences) and is represented as the average of the samples with standard error shown. Fold difference of gene expression was determined using the $\Delta\Delta C_t$ method ($2^{-(\Delta Ct, \text{experimental sample} - \Delta Ct, \text{reference sample})}$). $\Delta C_t = C_t$ (target gene) – C_t (endogenous control).

3.12.3 Quantitative PCR analysis of goldfish *kita* and *kitla* expressions in healthy goldfish tissues

Heart, blood, brain, gill, intestine, kidney, and spleen were harvested from four individual fish (n = 4) and RNA isolated using Trizol, and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit.

3.12.4 Quantitative PCR analysis of goldfish *kita* and *kitla* expressions in PKM cultures.

Cells from four individual fish (n = 4) were seeded at 2 mL/well in a 6-well culture plate at a concentration of 1 x10^6 cells/mL in complete NMGFL-15 medium. Cells were harvested at 0, 2, 4 and 8 days post culture using a cell scraper. Cell suspensions were centrifuged at 300 x *g* for 5 min to pellet cells and RNA was isolated using Trizol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit.

3.12.5 Quantitative PCR analysis of goldfish *kita* and *kitla* expressions in activated goldfish macrophages

Cells obtained from four goldfish PKM cultures (day 8 of cultivation) were pooled and seeded in 2 mL of complete NMGFL-15 medium at a cell concentration of 5 x 10^6 cells/mL. Day 8 PKM cultures were chosen as these represent cultures in which the majority of cells are mature macrophages [2, 3]. Cells were either treated with medium alone, 5 µg/mL of LPS (*E. coli* cat no. L 2630, Sigma) or 1:200 dilution of a heat-killed *A. salmonicida* stock for 24 hrs prior to RNA isolation with Trizol and was reverse transcribed into cDNA using

the Superscript II cDNA synthesis kit. Triplicate plates were run and analyzed using the 7500 Fast software. Fold difference compared to the untreated cells was calculated.

3.12.6 Quantitative PCR analysis of *kita* and *kitla* expressions in sorted goldfish macrophage subpopulations.

Cultured primary kidney macrophage cultures were sorted into early progenitor, monocyte or macrophage subpopulations based on size and internal complexity using a FACSCalibur flow cytometer (Becton Dickinson). RNA was isolated from the cell populations immediately after sorting using Trizol and RNA was reverse transcribed into cDNA using the Superscript III cDNA synthesis kit. Each bar is representative of the average of cells sorted from three individual fish (n = 3).

3.12.7 Quantitative PCR of transcription factors of R1 cells isolated from PKM cultures

R1 cells from day 0, day 2, or day 6 PKM cultures established from three fish at each time point were sorted on a FACS Aria flow cytometer. RNA was isolated using the RNA MicroPrep Kit (ZymoResearch) and reverse transcribed into cDNA using Superscript II enzyme. Values were normalized to the R1 day 0 control for each transcription factor.

3.12.8 Quantitative PCR of transcription factors of macrophage sub-populations

R1 progenitor cells from freshly isolated PKMs, R3 monocytes from day 2-4 of PKM cultivation, and R2 macrophages from day 6-8 of PKM cultivation were sorted based on size and internal complexity. RNA was isolated using the RNA MicroPrep kit and cDNA synthesized using the Superscript II enzyme. Each cell population was sorted from four fish. Data were normalized to the R1 progenitor cells.

3.12.9 Treatment of goldfish R1 progenitor cells with recombinant growth factors

Day 1, day 2 or day 3 PKM cells were collected and R1 cells sorted on a FACS Aria flow cytometer. Cultures established from 3-4 individual fish were used at each time point. Cells were washed 2 x in NMGFL-15, and re-suspended at a concentration of 3 x 10^5 cells/mL. To each well of a 6-well plate, 1 mL of cell suspension was added, followed by 1 mL of either medium, 200 ng/mL of rgKITLA, 200ng/mL of rgCSF-1, or a combination of 200 ng/mL of rgKITLA and 200 ng/mL of rgCSF-1. This resulted in a final concentration of 100 ng/mL of rgKITLA or 100 ng/mL of rgCSF-1. Cells were harvested at 0.5, 3 and 6 hours post treatment, RNA isolated using the RNA MicroPrep Kit (ZymoResearch), reverse transcribed to cDNA using Superscript II enzyme (Invitrogen). Values were normalized to the medium treated cells (negative control) for all transcription factors (n = 3-4).

3.12.10 Quantitative PCR of transcription factors in progenitor cells from *in vivo* challenged goldfish

3.12.10.1 Trypanosomes

Trypanosomes were washed twice in serum-free TDL-15 medium, enumerated using a haemocytometer and re-suspended to 1 x 10^9 trypanosomes/mL immediately prior to use in 3 days post infection (dpi) experiments or 6.25 x 10^7 trypanosomes/mL for use in 7 dpi experiments. The infection dose of 100 μL of 6.25 x 10^7 trypanosomes/mL (6.25 x 10^6 trypanosomes per fish) was selected based on previous studies in which all fish infected with this number of parasites became infected [8].

3.12.10.2 Aeromonas salmonicida A449

Aeromonas salmonicida A449 was heat killed by incubating at 60°C for 45 minutes in a water bath. The injection of fish with 1 x 10^9 heat-killed *A. salmonicida* was based on numbers used in previous literature in which authors injected with 1 x 10^8 live *Aeromonas* [9]. However, since heat-killed bacteria do not cause the same pathology as a live infection, I chose to increase the dose to 1 x 10^9 bacteria/fish.

3.12.10.3 In vivo challenge

Goldfish were injected i.p. with either PBS, 1 x 10^9 CFU of heat-killed *A. salmonicida* A449, 1 x 10^8 *T. carassii* (3 dpi challenge experiment), or 6.25 x 10^6 trypanosomes (7 dpi challenge experiment) in a volume of 100 μL. The 3 dpi or 7 dpi, goldfish were sacrificed and kidneys and spleens harvested. Kidneys from

fish at 3 dpi were dissociated as described above in section 1.3.2.1, and R1 cells sorted as described in section 1.5.1. Following cell sorting, R1s were centrifuged at 230 x *g* for 15 minutes and medium aspirated. RNA was isolated using the RNA MicroPrep Kit (ZymoResearch), and reverse transcribed into cDNA using the Superscript II enzyme (Invitrogen). For 7 dpi *T. carassii* challenged goldfish, RNA was isolated from the kidney and spleen tissue using Trizol (Invitrogen) and reverse transcribed into cDNA using Superscript II. Data were normalized for each transcription factor to that of the PBS-injected controls (n = 4).

3.12.11 Quantitative PCR analysis of goldfish *gcsfr* mRNA expression in goldfish tissues

Heart, blood, brain, gill, intestine, kidney, and spleen were harvested from four individual fish and RNA isolated using Trizol (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen)

3.12.12 Quantitative PCR expression of goldfish *gcsfr* mRNA in cells from PKM cultures.

In the first set of experiments, cells isolated from goldfish kidney from four individual fish were seeded at 2 mL/well in a 6-well plate at a concentration of 1 $x10^6$ cells/mL in complete NMGFL-15 medium. Cells were harvested at 0, 2, 4 and 6 days post culture. In the second set of experiments, PKM cultures were set up as described in section 1.3.3.1, and R1 gated cells consisting of mainly early progenitor cells were sorted using a FACS Aria Flow cytometer based on size and internal complexity at day 0, 2 and 6 post cultivation. RNA isolation was

performed using the RNA MicroPrep kit and cDNA synthesized using the Superscipt II enzyme.

3.12.13 Quantitative PCR analysis of *gcsfr* mRNA expression in cell populations.

Progenitor cells from freshly isolated primary kidney macrophage cultures were sorted based on size and internal complexity using a FACS Aria flow cytometer. Neutrophils from four individual fish were cultured overnight, and suspension cells used in this assay. Splenocytes and PBLs from four individual fish were isolated as described in section 1.4.1. RNA was isolated using Trizol and cDNA synthesis performed using the Superscript II enzyme. Data were presented as the mean $\pm$ SEM from four fish (n = 4).

3.12.14 Quantitative PCR analysis of *gcsfr* mRNA expression in stimulated neutrophils

In the first experiment, neutrophils from 5-6 individual fish were seeded into 6-well plates in 1 mL of complete NMGFL-15 medium at a concentration of 2 x 10^6 cells/mL. Cells were either treated with 1 mL of medium alone, or a mixture of calcium ionophore (100 ng/mL final concentration) phorbol esters (PMA, final concentration 10 ng/mL) and convacalin A (ConA 10 μg/mL final concentration) for 1, 3 or 6 hrs. In the second experiment, neutrophils from 5 individual fish were treated with medium or a 1:200 dilution of a heat-killed *A. salmonicida* for 3 hrs. Following incubation, medium was aspirated and RNA isolated using Trizol and cDNA synthesis performed using Superscript II enzyme.

3.13 Generation of recombinant protein expression constructs

3.13.1 Primers

All primers used in the generation of recombinant protein expression constructs can be found in Table 3.8.

3.13.2 Prokaryotic recombinant protein expression

3.13.2.1 Recombinant goldfish PROMININ extracellular loop-1 (rgPECL-1)

Primers were designed for cloning the rgPECL-1 into the pET151 vector (Invitrogen). Primers were used to set up a PCR using the conditions stated in section 1.9.4 using cDNA generated from the goldfish kidney as a template. The thermocycling program was as follows: an initial denaturation step at 94°C for 2 minutes followed by 31 cycles of 94°C for 30 seconds, 60°C for 20 seconds, 72°C for 2.5 minutes, and a final extension of 72°C for 10 minutes. The PCR product was visualized on a 1% agarose gel stained with ethidium bromide, and bands excised. Amplicons were gel purified and cloned into the pET151 vector according to the manufacturer's protocol. Plasmids were used to transform One Shot Top 10 *E.coli*, and colonies grown on LB + 100 μg/mL ampicilin plates overnight at 37°C. Positive colonies were identified by colony PCR and sequenced using pET151 vector specific primers to confirm sequence orientation and correct reading frame.

3.13.2.2 Recombinant goldfish Leukemia Inhibitory Factor (rgLIF)

The plasmid containing the insert for rgLIF was previously generated in our laboratory as described in [10].

3.13.3 Eukaryotic recombinant protein expression

3.13.3.1 Recombinant goldfish kit ligand a (rgKITLA)

Kit ligand A expression primers were designed for cloning into the pSECTag2B mammalian expression vector (Invitrogen) and PCR amplification of the expression insert was set up as follows: 60.75 μL DEPC treated water, 0.75 μL AccuPrime Pfx DNA polymerase (2.5 U/μL, Invitrogen), 7.5 μL of 10x AccuPrime PCR buffer (0.3 mM dNTPs, 1 mM $MgSO_4$ final concentrations), 0.3 μM sense and anti-sense expression primers, 1.5 μL goldfish kidney cDNA template. PCR amplification was conducted on an Eppendorf Mastercycler gradient thermal cycler and the thermocycling program consisted of a 2 min at 95°C denaturing step followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 68°C for 90 sec, and a final elongation step at 68°C for 15 min. Following this program, a 10:1 ratio of Taq was added to the reaction mixture and allowed to incubate at 72°C for 10 min to add 3' dATP overhangs to facilitate cloning the expression insert into the pCR2.1 Topo TA vector (Invitrogen) according to manufacturer's instructions. Positive colonies were identified by colony PCR and the isolated plasmid sequenced using the vector specific primers, M13 forward and reverse. Eight microlitres of pCR2.1 constructs containing the correct

expression insert or pSECTag2B vector (~300 ng/μL) were restriction digested with 1 μL of both HindIII and XhoI in 2.5 μL of 10X R buffer (Fermentas) and 12.5 μL of double distilled water. Digested expression insert and digested pSECTag2B vector were gel purified using the QIA gel extraction kit and ligated using T4 DNA ligase (Invitrogen) at 22^{o}C for an hour followed by 16^{o}C overnight according to manufacturer's protocols. Positive colonies were identified by colony PCR and sequenced using pSECTag2B vector specific primers T7 and BGH. In all cases, amplification and complete restriction digestion was confirmed by agarose gel electrophoresis.

3.13.3.2 Recombinant goldfish colony-stimulating factor-1 (rgCSF-1)

Goldfish colony-stimulating factor-1 (rgCSF-1) was amplified using gene specific primers (IDT, Table 3.8). PCR amplification of the expression insert was set up as follows: 60.75 μL DEPC treated water, 0.75 μL AccuPrime Pfx DNA polymerase (2.5 U/μL, Invitrogen), 7.5 μL of 10x AccuPrime PCR buffer (0.3 mM dNTPs, 1 mM $MgSO_4$ final concentrations), 0.3 μM sense and anti-sense expression primers, 1.5 μL goldfish CCL71 cDNA template (cDNA was synthesized using 5 μg of RNA). PCR amplification was conducted in an Eppendorf Mastercycler gradient thermal cycler and the thermocycling program consisted of a 6 min hot-start at 95^{o}C followed by 31 cycles of 94^{o}C for 20 sec, 59^{o}C for 30 sec, and 68^{o}C for 2 minutes, and a final elongation step at 68^{o}C for 7 min. Following this program, a 10:1 ratio of Taq was added to the reaction mixture and allowed to incubate at 72^{o}C for 10 min to add 3' dATP overhangs to facilitate cloning the expression insert into the pCDNA3.1 Topo TA vector

(Invitrogen) according to manufacturer's instructions. The amplicon was gel purified using the QIA Gel Extraction Kita (Qiagen) and cloned into the pCDNA3.1 mammalian expression vector (Invitrogen) according to manufacturers protocols. Constructs were screened by colony PCR using the vector specific primers T7 and BGH (Table 3.5) and plasmid from positive colonies were isolated using the QIA Miniprep Kit (Qiagen) and sequenced using an ET terminator cycle sequencing dye and run on a PE Applied Biosystems 377 automated sequencer. Sequences were analyzed using the 4peaks software (http://mekentosj.com/4peaks/) for correct orientation and reading frame.

3.13.3.3 Generation of the goldfish sCSF-1R/IpFcRγL fusion protein constructs.

The leader sequence and the two extracellular Ig domains of the soluble colony-stimulating factor-1 receptor (sCSF-1R, Accession number A4536524.1) were fused to the trans-membrane and intracellular signaling domain of the IpFcRyL chain (Accession number AF543420.1) using overlap extension PCR in a similar manner to that previously described in [11] and [4]. Briefly, the forward primer was designed against the 5' end of the sCSF-1R to include a SmaI restriction cut site, while the reverse primer was designed to have a 3' end that would overlap with the 5' end of the IpFcRy chain. The forward primer to the IpFcRγL chain was designed to have the 5' end overlap with the 3' end of the goldfish sCSF-1R primer, and the reverse primer was designed to have a SalI restriction cut site. Primers used for overlap extension PCR are listed in Table 3.8. To generate the desired products, PCR reactions were set up using Phusion

High-Fidelity DNA polymerase (Finnzymes) according to the manufacturer's specifications. To amplify sCSF-1R, goldfish kidney cDNA was used as the template. Amplification of the transmembrane and cytoplasmic tail of IpFcRγL was generated using a construct containing the full length IpFcRγL as the template [12]. Thermocycling conditions were 98°C for 90 seconds, followed by 30 cycles of 98°C for 15 seconds, 60°C for 20 seconds, 72°C for 60 seconds, followed by a final extension step of 72°C for 10 minutes. PCR products were run on a 1% agarose gel, corresponding bands excised, gel purified, and cloned and sequenced to confirm identity. To create the goldfish sCSF-1R/IpFcRγL fusion construct, overlap extension PCR was performed. Briefly, 100 ng of the purified PCR products were used as a template in the overlap extension PCR. As with the generation of the initial PCR products, Phusion High-Fidelity DNA polymerase was used in the generation of the fusion construct. Thermocycling conditions were as follows: 98°C for 2 minutes followed by 35 cycles of 98°C for 1 minute, 55°C for 30 seconds, 72°C for 2 minutes, with a final extension step of 72°C for 10 minutes. The resulting PCR product was cloned into the pDISPLAY vector (Invitrogen) according to manufacturer's instructions, and sequenced with T7 sense and BGH antisense primers to confirm the sequence was in frame.

RBL2H3 cells expressing the IpLITR/IpFcRγL were a kind gift from Dr. James L. Stafford, University of Alberta. The IpLITR/IpFcRγL fusion construct was used as a positive control and was generated previously as described in [4].

3.14 Production of recombinant proteins

3.14.1 Prokaryotic systems

3.14.1.1 Recombinant goldfish Leukemia Inhibitory Factor (LIF)

The rgLIF/SUMO Plasmids maintained in Mach 1 cells were purified using the QIAprep Miniprep kit (Qiagen) according to manufacturers instructions and used to transform BL21 *E. coli*. Transformed BL21 *E. coli* were grown in LB containing kanamycin (50 μg/mL) overnight at 37°C, 250 rpm. This overnight culture was used to inoculate LB + kanamycin medium at a 1:10 dilution. Bacteria were grown under the same conditions and following 2 hrs of growth, 1 mM of IPTG was added to induce the expression of the recombinant protein. Cultures were harvested four hours following induction, and centrifuged for 15 minutes at 10,000 x *g*. Cell pellets were retained and stored -20°C, until purification.

3.14.1.2 Recombinant goldfish PROMININ *extracellular loop-1 (rgPECL-1)*

The PROMININ extracellular loop 1/pET 151 construct was used to transform BL21 star *E. coli,* and were grown overnight in 10 mL of LB containing ampicillin (100 μg/mL). This starter culture was used for pilot expression studies to determine the optimal concentration of IPTG for induction and the time required for protein expression before harvesting the bacteria containing the recombinant protein. An optimal concentration of 0.1 mM IPTG, and 4 hours post IPTG induction were chosen and used for large-scale expression of the recombinant protein. A 1:10 dilution of an overnight starter culture was used to inoculate the LB + 100 μg/mL amplicillin medium used for protein expression.

Bacteria were centrifuged (10, 000 x *g*) and the bacterial pellet frozen overnight at -20°C.

3.14.2 Eukaryotic systems

3.14.2.1 Recombinant goldfish kit ligand a (rgKITLA)

Pilot protein expression studies for rgKITLA were performed by seeding HEK293T cells at a concentration of 4 x 10^5 cells/well in a 6 well plate in a total volume of 2 mL of complete DMEM. Cells were grown at 37°C at 5% CO_2 and upon becoming ~50% confluent, which usually occurred after 48 hrs, they were transfected with 1.5 μg of the pSECTag2B/ KITLA expression plasmid using TurboFect (Fermentas) according to manufacturer's instructions. Cells were grown for an additional 2 days at which time both supernatants and cell lysates were harvested to test for the presence of recombinant protein. Presence of recombinant protein was assessed by Western blot using a 1:1500 dilution of a mouse anti-His antibody (Sigma) followed by a secondary goat anti-mouse alkaline phosphatase (AP) antibody at a 1:3000 dilution (Biorad) to detect the polyhistidine tag located at the C-terminus of the recombinant protein. Alkaline phosphatase activity was detected by using the developing reagents NBT and BCIP as per manufacturer's instructions (Biorad).

Scale up expression studies consisted of seeing HEK293T cells into 75 cm^2 flasks, allowing them to reach 50% confluence and then performing a scaled up transient transfection protocol using Turbofect (Fermentas). Following transfection, transfected HEK293T cell cultures were allowed to grow at 37°C,

5% CO_2 for an additional 5 days and culture supernatants collected and stored at 4°C until purification.

3.14.2.2 Recombinant goldfish colony-stimulating factor-1 (rgCSF-1)

Pilot protein expression studies for rgCSF-1 was performed by seeding HEK 293T cells at a concentration of 4 x 10^5 cells/well in a 6 well plate in a total volume of 2 mL of complete DMEM. Cells were grown at 37°C at 5% CO_2 and upon becoming ~50% confluent, which usually occurred after 48 hrs, they were transfected with 1.5 μg of the rgCSF-1/pCDNA3.1 expression plasmid using TurboFect (Fermentas) according to manufacturer's instructions. Cells were grown for an additional 2 days at which time both supernatants and cell lysates were harvested to test for the presence of recombinant protein. Presence of recombinant protein was assessed by Western blot using a 1:5000 dilution of a mouse anti-V5-HRP antibody to detect the polyhistidine tag located at the C-terminus of the recombinant protein.

For scale up expression, the CSF-1 construct was transfected into 50-60% confluent CHO cells in T-75 cm^3 vented flasks using Turbofect (Fermentas) according to the manufacturers protocol. CHO cells were grown at 37°C in the presence of 5% CO_2 in complete DMEM. The medium was collected every 5-6 days following transfection for 2-3 weeks, cleared of suspension cells by centrifugation at 230 *g* for 10 minutes, and stored at 4°C prior to purification of secreted recombinant goldfish CSF-1 (rgCSF-1).

3.14.3 Transfection and selection of RBL2H3 cells expressing goldfish sCSF-1R/IpFcRγL fusion protein

RBL2H3 cells were seeded into a 24-well cell culture plate at a concentration of 1.5 x 10^5 cells/well in 500 μL of MEM medium containing 10% FBS. The following day, the medium was replaced with 250 μL of Opti-MEM for transfection of the cells. The adherent RBL2H3 cells were transfected with 1 μg of plasmid containing the sCSF-1R/IpFcRyL fusion protein using Xfect (Clontech) according to the manufacturer's protocol. Four hours following transfection, 750 μL of complete MEM was added. Cells were incubated for an additional 48 hours at 37^{o}C in 5% CO_2 to allow cells to become fully confluent. Following incubation, cell media were replaced with complete MEM containing 800 μg/mL of G418 to select for clones positive for the plasmid. Medium was replaced with fresh medium every 3-4 days until cells were confluent. Following selection of cells expressing the construct, cultures were maintained in complete MEM with 400 μg/mL of G418.

3.15 Purification of recombinant proteins

3.15.1 Prokaryotic expression systems: rgLIF and rgPECL-1

The rgLIF or rgPECL-1 was purified from cell lysates generated from 250 mL of bacterial culture using MagneHis beads (Promega). Cell lysates were lysed in 50 mL of 1x MagneHis lysis buffer diluted in denaturing wash buffer (100 mM HEPES, 10 mM imidazole, 7.5 M urea, pH 7.5, in the presence of a protease inhibitor cocktail (Calbiochem) for 1 hour with end-over-end mixing. Cell lysates were incubated with 500 μL of MagneHis beads for 2 hours at room temperature

with end-over-end mixing. The recombinant proteins were purified under denaturing conditions, and MagneHis beads were thoroughly washed with binding/wash buffer (7.5 M urea, 100 mM HEPES, 10 mM imidazole, pH 7.5) prior to elution with elution buffer (7.5 M urea, 100 mM HEPES, 500 mM imidazole, pH 7.5). Following purification, proteins were renatured overnight by the dropwise addition of the purified protein into cold (4°C) renaturation buffer (50mM Tris, 1 mM EDTA, 0.1 M L-arginine, 1 mM reduced glutathione, 8 mM oxidized glutathione, pH 8.2) with gentle agitation. The protein was concentrated using Snakeskin tubing and dialyzed against 1x PBS for 24-48 hrs. The protein was applied to an EndoTrap column (Lonza) following the manufacturers instructions to remove endotoxins, and an LAL assay used to confirm removal of endotoxin (Lonza). Total protein concentration was determined using a BCA assay with bovine serum albumin (BSA) as the standard curve (Pierce). The identity of the purified rgPECL-1 was confirmed using mass spectrometry.

3.15.2 Recombinant goldfish kit ligand a (rgKITLA)

Supernatants collected from transfected HEK 293T cells were adjusted with a 1:10 dilution using a 10x NiNTA binding buffer (500 mM Tris, 1.5 M NaCl, 200 mM imidazole, pH 8.0) and incubated with NiNTA beads over a 4-6 hr period at 4°C with intermittent agitation. The supernatants containing NiNTA beads were poured through a column to collect the NiNTA agarose beads and were subsequently washed with three column volumes of each of 20 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM imidazole elution buffer (300 mM NaCl, 50 mM Tris, pH 8.0). All binding and elution buffer solutions were first treated with a

protease inhibitor cocktail (Calbiochem) and all steps were performed at 4°C to minimize protein degradation. Elution fractions were tested for presence of protein by separation on a 12% SDS-PAGE gel under reducing conditions and the proteins were transferred to a 0.2 μm nitrocellulose membrane (BioRad). The nitrocellulose membrane was blocked for 30 min with 0.5 % BSA and then incubated overnight at 4°C with a monoclonal mouse anti-His antibody (Sigma) to detect the six histidine tag at the C-terminus of the recombinant protein. Membranes were washed with Tris-buffered saline containing 0.025% Tween-20 and incubated with a goat anti-mouse IgG alkaline phosphatase conjugated antibody (BioRad). Membranes were developed using 5-bromo-4-chloro-3-indolyl phosphatase (BCIP) and nitro blue tetrazolium (NBT). Fractions containing the purified protein were pooled, concentrated and dialyzed against 50 mM Tris, pH 8.0. Purified recombinant kit ligand was filter sterilized, quantified using a bicinchroninic acid (BCA) kit with bovine serum albumin as the standard curve as per manufacturer's specifications (Biorad), and stored at 4°C until use. Mass spectrometry was performed to confirm the identity of the recombinant protein.

3.15.3 Recombinant goldfish colony-stimulating factor-1 (rgCSF-1)

CHO cell-conditioned supernatants were adjusted to 20 mM imidazole and 100 mM HEPES, required for protein binding to beads. MagneHis (Promega) beads were added to the adjusted solution and incubated overnight at 4°C. MagneHis beads were collected using the magnetic stand provided, and beads washed 5 x with binding buffer (20 mM imidazole, 100 mM HEPES), followed by protein elution from the beads in 1 mL fractions using the provided MagneHis

elution buffer according to manufacturers instructions. Buffer exchange from MagneHis elution buffer to 1X PBS was performed using Zeba Spin Desalting Columns (Thermo Scientific) according to specifications. The amount of protein was determined using the Micro BCA Protein Assay Kit (Thermo Scientific) employing bovine serum albumin as the standard curve. The solution containing rgCSF-1 was filter sterilized (0.22 μm) and stored at 4^{o}C until use. Mass spectrometry was used to confirm the identity of the recombinant protein (Mass Spectrometry Facility, Department of Chemistry, University of Alberta).

3.16 Immunodetection of recombinant proteins

3.16.1 Western blot analysis

Recombinant proteins were separated by PAGE gels under native or denaturing and reducing conditions. Proteins were transferred to a 0.2 μm nitrocellulose membrane (Biorad) and blocked for 0.5 hr at room temperature. Membranes were incubated with the appropriate antibody for 3 hours at room temperature, membranes were washed, and in some cases, a secondary antibody was applied to the blot for 1 hour at room temperature. The membranes were developed using BCIP and NBT (BioRad) for alkaline phosphatase (AP) conjugated antibodies, or with the ECL Western Blotting Substrate (Pierce) to detect horse-radish peroxidase (HRP) conjugated antibodies according to the manufacturer's protocol. An anti-HIS antibody (1:1500) followed by a goat anti-mouse-AP antibody (1:3000) were used to detect rgPECL-1, rgLIF and rgKITLA. In some cases, rgPECL-1 was recognized by the anti-V5-HRP antibody (1:5000),

and rgKITLA was recognized by the anti-myc-HRP antibody (1:3000). The rgCSF-1 protein was recognized by the anti-V5-HRP (1:5000) antibody.

For detection of rgPECL-1 or rgKITLA with anti-rgPECL-1 or anti-rgKITLA antibodies, respectively, nitrocellulose membranes were incubated with the primary rabbit polyclonal antibody overnight at 4°C. Membranes were incubated with a goat anti-rabbit secondary antibody for 1 hour at room temperature. Nitrocellulose membranes were washed after each antibody incubation and developed.

3.16.2 Immunoprecipitation

To determine whether the polyclonal rabbit antibodies could be used in immunoprecipitation studies, the ability of anti-rgKITLA to pull down rgKITLA, or anti-rgPECL-1 to pull down rgPECL-1 from solution was assessed. Recombinant protein was incubated with anti-recombinant *Trypanosoma carassii* calreticulin (anti-CRT) or varying concentrations (0 μg, 1 μg, 2 μg, 4 μg and 8 μg) of protein speciic antibody for 2 hours. To the protein-antibody mixture, Protein G beads were added and incubated overnight. Following incubation, the supernatant was removed from the beads (S) and the beads were washed to remove any unbound protein. SDS-PAGE loading buffer was added to the beads and boiled to dissociate antibody-protein complexes from the beads (B). The supernatant was run on a reducing gel along with a well containing recombinant protein as a loading control. Following transfer to a nitrocellulose membrane, blots were probed with an anti-myc HRP antibody that recognizes the myc epitope on the C-

terminus of the rgKITLA protein or anti-V5-HRP antibody that recognizes the V5 epitope located at the N-terminus of rgPECL-1.

3.17 Production of polyclonal antibodies to recombinant proteins

3.17.1 Anti-rgPECL-1 IgG

Recombinant goldfish PROMININ extracellular domain-1 was used to immunize two rabbits. Rabbits were injected with 200 μg of the purified recombinant protein in 750 μL of PBS and an equal volume of Freund's Complete Adjuvant (FCA). The three booster injections were performed using 200 μg of recombinant protein in Freund's Incomplete Adjuvant (FIA).

3.17.2 Anti-rgKITLA IgG

Recombinant goldfish KITLA was used to immunize one rabbit. The rabbit was injected with 125 μg of the purified recombinant protein in 750 μL of PBS and an equal volume of Freund's Complete Adjuvant (FCA). The three booster injections were performed using 100 μg of recombinant protein in Freund's Incomplete Adjuvant (FIA).

3.17.3 Anti-recombinant soluble CSF-1R (sCSF-1R)

The identification, expression and purification of a recombinant sCSF-1R and the production of a rabbit polyclonal antibody against the sCSF-1R were performed as previously described in [13].

3.17.4 Affinity purification of polyclonal antibodies

Ice-cold saturated ammonium sulphate (SAS) was added to rabbit serum through a drop-wise addition with continuous agitation to a final percentage of 45% (v/v) SAS. This solution was rocked overnight at 4°C prior to centrifugation (30 min at 10,000 x *g*). The supernatant was aspirated, and the pellet re-suspended in 20 mM sodium phosphate buffer, pH 7.2. This solution was dialyzed against 4 L of 20 mM sodium phosphate buffer pH 7.2 overnight to remove excess salts prior to application to a Protein A column (GE Healthcare) following manufacturers instructions. Briefly, the solution was applied to the column, followed by washing with 10 column volumes of 20 mM sodium phosphate buffer, pH 7.2. Citric acid buffers of decreasing pH were applied to the column to elute the bound IgG from the column. The addition of 1M Tris, pH 9 was added to each elution fraction to neutralize the acidity of the solution and prevent antibody denaturation. Fractions containing rabbit IgG were pooled and dialyzed against 1x PBS pH 7.4 for 72 hours with multiple buffer exchanges. Following dialysis, a BCA assay (Pierce) was performed according to manufacturers directions to determine protein concentration. Affinity purified antibodies were filter sterilized (0.22 μm), and stored at -20°C until use.

3.18 Detection of recombinant protein interactions

To determine whether rgKITLA formed homodimers in solution, 100 μg of protein was incubated in 1 x PBS for 1 hour in the presence or absence of 100 μg of BSA. A cross-linking solution consisting of bis (sulfosuccinimidyl) suberate (BS^3, Pierce) was added for 15 minutes, and the reaction stopped according to

the manufacturer's instructions. The reaction was run on a reducing SDS-PAGE gel, transferred to a nitrocellulose membrane (Bio-Rad), and probed with an anti-myc-HRP antibody. The membrane with incubated with ECL substrate (Pierce) and exposed to film.

3.19 Immunofluorescence staining of goldfish cell populations

3.19.1 Immunostaining of sCSF-1R expressing RBLs

RBL cells transfected with the sCSF-1R/IpFcR chain or IpLITR/IpFcRγL were harvested using harvest buffer (1.5 mM EDTA, 135 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4), put on ice, and then washed twice with ice-cold FACS buffer (1x PBS, 5% FBS, 0.05% sodium azide). Cells were re-suspended to a concentration of 1 x 10^7 cells/mL, and 100 μL used for each treatment. Cells were incubated with either 100 μL of mouse IgG3 antibody (20 μg/mL), mouse anti-HA antibody (20 mg/mL), SPF rabbit IgG (25 μg/mL), or the anti-sCSF-1R IgG (25 μg/mL) for 30 minutes on ice. Cells were washed twice with FACS buffer, and re-suspended in 50 μL of goat anti-mouse IgG-PE (10 μg/mL) goat anti-rabbit IgG-PE (10 μg/mL) for 30 minutes. Cells were washed twice with FACS buffer prior to analysis on a Cell Lab Quanta SC flow cytometer (Beckman Coulter).

3.19.2 Immunostaining of catfish B-cells

Catfish 3B11 B-cells were washed twice in ice-cold FACS buffer, and re-suspended at a concentration of 1 x 10^7 cells/mL. One hundred microlitres containing 1 x 10^6 cells were used for each treatment. Cells were then incubated

with the SPF rabbit IgG (isotype control), the rabbit anti-sCSF-1R IgG, or 9E1 hybridoma supernatant (positive control, binds to surface IgM on catfish B-cells). Cells were then washed twice in FACS buffer, and 50 μL of the goat anti-rabbit IgG-PE secondary antibody (10 μg/mL) or goat anti-mouse IgG-PE (10 μg/mL) was added to the re-suspended cells for 30 minutes on ice. Cells were washed twice with FACS buffer prior to analysis on the FACS Calibur flow cytometer (Becton Dickinson).

3.19.3 Immunostaining of goldfish CCL71 cells

CCL71 cells were removed from confluent cell cultures using a cell scraper. Cells were washed twice in FACS buffer prior to addition of 100 μL of either SPF rabbit IgG (25 μg/mL) or rabbit anti-sCSF-1R (25 μg/mL) for 30 minutes on ice. Following incubation with the primary antibody, cells were washed twice in FACS buffer, incubated with 50 μL of goat anti-rabbit IgG-PE (10 μg/mL) for 30 minutes on ice, washed twice with FACS buffer, and analyzed for fluorescence on a FACS Calibur flow cytometer (Becton Dickinson).

3.19.4 Rabbit anti- sCSF-1R staining of cells from goldfish tissue mononuclear cell suspensions and PKMs.

In the first set of experiments, mononuclear cell preparations (section 1.4.2) or freshly isolated PKM cells, or cells from Day 1, Day 2, Day 3, or Day 4 PKM cultures (n = 7) were washed twice (1 min, 5,000 x *g*) with ice cold FACS buffer and re-suspended to a concentration of 1 x 10^7 cells/mL, and one million cells were used per treatment. Treatments consisted of cells alone, cells without

primary antibody and labeled with secondary antibody, or cells incubated with 100 μL of a 25 μg/mL solution of affinity purified specified pathogen free rabbit IgG (isotype control) or affinity purified rabbit anti recombinant sCSF-1R IgG. Cells were incubated with the primary antibody for 30 min on ice, washed twice (1 min at 5,000 x *g*) with FACS buffer, and incubated for an additional 30 min on ice with 50 μL of 10 μg/mL goat anti-rabbit IgG-PE (Beckman Coulter) secondary antibody. Cells were washed twice (1 min, 5,000 x *g*) with FACS buffer prior to analysis on the FACS Calibur flow cytometer (Becton Dickinson), n = 7.

In the second set of experiments, PKM cells from day 0 or day 4 cultures were split into two groups consisting of acid treated, and non-acid treated cells (n = 4). Cells were washed twice with ice-cold FACS buffer and re-suspended to a concentration of 1 x 10^7 cells/mL. One million cells in 100 μL were used for each treatment. Prior to staining with the anti-sCSF-1R antibody or the SPF rabbit IgG isotype control, cells were incubated with 1x PBS (pH 7.4) or a dilute acid solution (50 mM glycine in 1x PBS, pH 3.5) for three minutes on ice to remove any molecules bound to the surface of the cells. Following acid treatment, the acid was neutralized with a half volume of 0.5 M HEPES solution in PBS, pH 7.4. This acid treatment procedure was adapted from Cubellis et al. [14]. Cells were centrifuged and washed twice with FACS buffer. This was followed by incubation with the primary and secondary antibodies as detailed above in this section. Cells were analyzed on the FACS Calibur flow cytometer. A small sub-sample of cells was assessed for viability using Trypan blue to ensure acid treatment was not deleterious to the cells.

3.20 Cell bioassays

3.20.1 Flow cytometry analysis of fish cells

3.20.1.1 Primary kidney macrophages

Primary kidney macrophages were analyzed on a FACS Calibur flow cytometer based on forward scatter (size) and side scatter (internal complexity). The flow cytometer parameters were previously established in our lab and were as follows: forward side scatter: E0, AmpGain 1.05, side scatter: voltage 455V, AmpGain 1.00.

3.20.1.2 Primary kidney neutrophils

Primary kidney neutrophils were analyzed on a FACS Calibur flow cytometer. Cells were analyzed based on forward scatter (size) and side scatter (internal complexity). The flow cytometry parameters set on the FACS Calibur flow cytometer used to analyze the cells are as follows: forward light scatter (size): voltage E00, AmpGain 1.93 side scatter (internal complexity): voltage 472, AmpGain 1.00

3.20.2 Primary kidney macrophage chemotaxis assay

Cells from either day 2 or day 6-8 PKM cultures were washed twice in incomplete NMGFL-15 and adjusted to a final concentration of 1 x 10^6 cells/mL. The chemotaxis assay was performed using blind well leucite chemotaxis chambers (Nucleoprobe Corp.). Two hundred microlitres of recombinant goldfish KITLA, at concentrations ranging from 0.1 ng/mL to 1000 ng/mL, was added to the bottom well of the chemotaxis chambers. To the top chamber, 100 μL of PKM

(1×10^5) was added plus 100 μL of incomplete medium. The top and bottom chambers were separated by a 5 μm pore size polycarbonate membrane filter (Neuroprobe). Negative controls consisted of medium alone and the positive control was 10 ng/mL of recombinant goldfish leukemia inhibitory factor (rgLIF) in incomplete NMGFL-15. The chemokinesis control consisted of 100 ng/mL of recombinant goldfish KITLA placed in both the upper and lower chambers of the chemotaxis apparatus.

For chemotaxis experiments the incubation period was 4 hrs at 20°C. Following incubation the cell suspensions were carefully aspirated from the top chamber and the filters removed and applied bottom side up on a microscope slide. Filters were fixed in methanol for 1 minute and then stained with Hematoxylin Solution, Gill's No. 3 (Sigma) for 1 min. Chemotactic activity was determined by counting the total number of cells found on the underside of the polycarbonate filters in 20 random fields of view under oil immersion (100x). PKM were established from four individual fish (n = 4).

3.20.3 Measurement of early progenitor cell proliferation

Day 2 primary kidney macrophage cultures were washed twice with incomplete NMGFL-15 to remove serum proteins and adjusted to a concentration of 2×10^5 cells/mL. Fifty microlitres of this cell suspension was added to each well of a 96 well plate, followed by the addition of 50 μL of treatment in incomplete NMGFL-15. Treatments consisted of incomplete NMGFL-15 (negative control), cell conditioned medium (CCM, positive control) and varying concentrations of recombinant KITLA at final concentrations of 1000 ng/mL, 100

ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL, and 0.01 ng/mL. Cell proliferation was assessed using the BrdU colorimetric assay (Roche). Briefly, 15 μM of BrdU labeling reagent was added to each well 24 hrs prior to development and plates were kept at 20°C. Proliferation of cells was measured on days 0, 2, 4 and 6 and developed according to the manufacturer's protocols. Plates were read at an absorbance of 450 nm using a microplate reader (VersaMax). Well values obtained for the cells alone group in incomplete NMGFL-15 medium were subtracted from all other wells to control for cell proliferation due to production of endogenous growth factors.

3.20.4 Flow cytometry analysis of PKM cultures in the presence of recombinant goldfish KITLA

Day 2 PKM cultures were washed twice with incomplete NMGFL-15 and seeded at a concentration of 1 x 10^6 cells/well in 2 mL of medium. Cells were treated with medium alone, cell-conditioned medium (CCM) or 100, 10, 1, and 0.1 ng/mL of recombinant goldfish KITLA. Plates were incubated at 20°C and samples analyzed 0, 2, 4, 6 and 8 days post treatment using FACSCalibur flow cytometer (Becton Dickinson) set to parameters as described in section 1.19.1.1.

3.20.5 Primary kidney macrophage nitric oxide assay

The Griess reaction [15] was used to indirectly determine nitric oxide production by fish macrophages. One hundred thousand cells in 50 μL of complete NMGFL-15 medium were added to individual wells of a 96 well plate, followed by 50 μL of treatments also in complete NMGFL-15 medium. Plates

were incubated for 48 or 72 hours at 20°C. Following incubation, plates were centrifuged at 230 x *g* and 75 μL of cell media transferred to a new 96-well plate. One-hundred microlitres of 1% sulfanilamide (dissolved in 2.5% H_3PO_4) was added to each well, followed by 100 μL of 0.1% N-naphthyl-ethylenediamine (dissolved in 2.5% H_3PO_4). The reaction was allowed to proceed for two minutes before plates were read using the VersaMax micro plate reader (Molecular Devices) and Softmax Pro 5 software at a wavelength of 540 nm. The concentration of nitrate produced by individual samples was determined using a standard curve that was generated using sodium nitrate standard curve.

3.20.6 Neutrophil degranulation assay

Degranulation assays were performed based on previous protocols developed in bovids and fathead minnows [12, 18]. Briefly, neutrophils cultured overnight were re-suspended in Hank's balanced salt solution with calcium and magnesium, but without phenol red. Neutrophils from five fish were pooled and 50 μL of $2x10^6$ cells/mL were seeded into each well of a 96 well plate. Fifty μL of $HBSS^{Ca2+/Mg2+}$ with or without 5 μg/mL of cytochalasin B was then added to the wells. Treatments were applied to the cells in a volume of 50 μL, re-suspended in HBSS with calcium and magnesium, without phenol red. Cells were incubated for 30 minutes at room temperature. To normalize the assays for the varying amounts of myeloperoxidase within neutrophils and variability from plate to plate, 100 μL of 2% Hexadecyltrimethylammonium bromide (Sigma) was added to 50 μL containing 2 x 10^6/mL of neutrophils to lyse the cells in order to measure total peroxidase activity. After incubation, 50μL of 2.5 mM 3,3'5,5'-tetramethylbenzidine (TMB) was added to each well followed by 50 μL of 5mM

H_2O_2. The reaction proceeded for 2 minutes at which time 50 μL of 2 M sulphuric acid was added to stop the reaction. Plates were centrifuged at 400 x *g* for 10 minutes and 200 μL of the supernatant from each well transferred to a new 96-well plate. Optical density was read at 405 nm using a VersaMax micro plate reader (Molecular Devices) and Softmax Pro 5 software.

For all degranulation assays, the following formula was used to calculate % of total peroxidase activity:

$$\%\text{total peroxidase activity} = \frac{(\text{Experimental - control})}{(\text{Total peroxidase - control})} \times 100\%$$

3.20.7 Neutrophil respiratory burst assay

3.20.7.1 Mitogens

Neutrophils were washed twice with serum free NMGFL-15 medium, and re-suspended to a concentration of 2.5 x 10^6 cells/mL. Four-hundred microlitres of the cell suspension was added to each 5 mL polystyrene tube and allowed to rest for 1 hour prior to stimulation. Cells were loaded with 1μl of 29 mM dihydrorhodamine (DHR) for 5 minutes at the end of the rest period. Following cell loading, cells were stimulated for thirty minutes or one hour with 100μL of the desired treatment. Negative and positive controls were: HBSS $^{Ca2+/Mg2+}$ or medium alone, or hydrogen peroxide, respectively. Fluorescence was measured by flow cytometry using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson). The flow cytometry parameters set on the FACS Calibur flow cytometer used to analyze the cells are as follows: forward light scatter (size): voltage E00, AmpV 1.93 side scatter (internal complexity): voltage 472, AmpV

1.00 FL1 428, FL2 458, FL3 640. Ten thousand events per sample were analyzed.

3.20.7.2 With live A. salmonicida A49

The preparation of neutrophils and treatments were the same as described above in section 3.7.2.2. For the induction of the respiratory burst response by *A. salmonicida,* neutrophils were treated with *A. salmonicida* suspended in incomplete medium at concentrations ranging from 1 x 10^6 to 2 x 10^9/ml resulting in *A. salmonicida* to neutrophil ratios of 0.1:1 to 200:1, respectively. The negative control was 100 μL of incomplete medium, and the positive controls were either hydrogen peroxide or 100 μL of PMA at a final concentration of 1 μg/mL. After 1 hour of incubation, the fluorescence emitted by the neutrophil cells was measured using flow cytometry using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson) using the following parameters: forward light scatter (size): voltage E00, AmpV 2.0 side scatter (internal complexity): voltage 477, AmpV 1.00 FL1 551, FL2 489, FL3 597. These parameters were optimized to ensure that non-labeled neutrophils, non-labeled *A. salmonicida* and labeled *A. salmonicida* could be distinguished from loaded neutrophils (both non-stimulated and stimulated neutrophils), with an overlap of less than 3% of total cells in all cases. The *A. salmonicida* was gated and excluded from analysis in order to obtain an accurate measurement of neutrophil respiratory burst induced by the bacterium. Because of the large number of bacteria in some sample wells, events were collected for 30 seconds where a minimum of 30,000 neutrophil events were collected per sample.

3.20.8 Neutrophil chemotaxis assay

Neutrophils were washed twice in incomplete NMGFL-15 medium and adjusted to a final concentration of 1 x 10^6 cells/mL. Treatments were also prepared in incomplete NMGFL-15 medium. The chemotaxis assay was performed using blind well leucite chemotaxis chambers (Nucleoprobe Corp.). One-hundred microlitres of the desired treatment plus 100 μL of incomplete medium were added to the bottom well of the chemotaxis chambers, and the bottom chamber was separated from the top chamber using 5 μm pore size polycarbonate membrane filters (Neuroprobe). To the top chamber, 100 μL of neutrophil suspension was added plus 100 μL of incomplete medium. Negative controls consisted of medium alone and the positive control was 10 ng/mL fMLP (N-formyl-methionyl-leucyl-phenylalanine). The chemokinesis control consisted of equal amounts of the treatment in both the upper and lower chambers of the chemotaxis apparatus. The chemotaxis assay was incubated at room temperature for one hour. Following incubation, the cell suspension was aspirated from the top well, and the filter removed. The top-side of the filter (facing the cell suspension) was gently wiped with a Kim-wipe to remove any cells that had settled onto the filter during the incubation and had not actively migrated through the pores, across the membrane. Cells that had actively migrated to the bottom side of the filter were fixed in methanol for 1 minute, rinsed in distilled water, and stained with Gill's Solution 3 for five minutes to visualize cells. Filters were rinsed under tap water for one minute, and mounted, bottom side up, onto glass slides. Chemotactic activity was determined by counting the total number of cells found on the underside of the polycarbonate

filters in 20 random fields of view under oil immersion (100X). Duplicate filters were used in each experiment.

3.20.9 Neutrophil nitric oxide assay

Nitric oxide production by day 1 neutrophils from individual fish was measured indirectly using the Griess reaction as described in section 1.19.5. Treatments consisted of complete medium, or a 1:35 dilution of heat-killed *A. salmonicida* A449, grown to lag phase, in incomplete NMGFL-15 medium. Cells were incubated with treatments for 48 or 72 hours prior to detection of micromolar nitrate, n = 4.

3.21 Statistical analysis

For qPCR data, statistical analysis was performed on the delta Ct values and included unpaired and paired t-tests and one-way analysis of variance (ANOVA) with a Dunnett's multiple comparisons post hoc test or Tukey's post hoc test. For cell based assays, unpaired t-tests and one-way analysis of variance (ANOVA) with a Dunnett's or Tukey's post hoc test were used to determine significance between treatment groups. All data were tested for normality and were found to have a normal distribution. Significance was set at $P < 0.05$ and $P < 0.08$.

Table 3.1: Composition of one litre (1 L) of incomplete NMGFL-15 medium

Component	Amount
HEPES	3.5 g
KH_2PO_4	0.344 g
K_2HPO_4	0.285 g
NaOH	0.375 g
$NaHCO_3$	0.17 g
10x Hank's Balanced Salt Solution	40 mL
MEM amino acid solution (50X)	12.5 mL
MEM non-essential amino acid solution (100X)	12.5 mL
MEM sodium pyruvate solution (100 mM/100X)	12.5 mL
MEM vitamin solution	10 mL
Nucleic acid preparation solution	10 mL
2-Mercaptoethanol solution	3.5 μL
GFL-15 medium*	500 mL
L-glutamine	0.2922 g
Insulin	0.005 g
Milli-Q water	Fill to 1 L

pH to 7.4 and filter sterilized using a 0.2 μm filter. Stored at 4°C.

*GFL-15 medium is made by mixing equal volumes of Leibowitz's L-15 medium with Delbecco's Modified Eagle Medium (DMEM) with phenol red. GFL-15 medium is filtered using a 0.2 μm filter and stored at 4°C.

Table 3.2: Composition of nucleic acid precursor solution

Component	Amount
Adenosine	0.67 g
Cytidine	0.061 g
Hypoxanthine	0.034 g
Thymidine	0.061 g
Uridine	0.061 g
Milli-Q water	100 mL
Do not filter, store at 4^{o}C.	

Table 3.3: Composition of 10x Hanks Balanced Salt Solution (HBSS)

Component	Amount
KCl	2 g
KH_2PO_4	0.3 g
NaCl	40 g
Na_2HPO_4-$7H_2O$	0.45 g
D-glucose	5 g
Phenol Red	0.05 g
Milli-Q water	Up to 500 mL
Filter sterilized with a 0.2 μm filter, stored at 4°C.	

Table 3.4: Composition of TDL-15 medium

Component	Amount
10x Hank's Balanced Salt Solution	40 mL
MEM amino acid solution (50X)	12.5 mL
MEM non-essential amino acid solution (100X)	12.5 mL
$NaHCO_3$	1.26 g
NaOH (1N)	0.15 mL
MEM sodium pyruvate solution (10 mM)	12.5 mL
Nucleic acid preparation solution	10 mL
L-glutamine	0.2922 g
Gentamycin solution	1 mL
MEM vitamin solution	10 mL
2-Mercaptoethanol solution (50 mM)	1 mL
HEPES	2 g
Insulin	0.005 g
GFL-15 medium*	500 mL
Milli-Q water	Fill to 1 L

pH to 7.2 and filter sterilize using a 0.2 μm filter. Store at 4°C.

*GFL-15 medium is made by mixing equal volumes of Leibowitz's L-15 medium with Delbecco's Modified Eagle Medium (DMEM) with phenol red. GFL-15 medium is filtered using a 0.2 μm filter and stored at 4°C.

Table 3.5: Vector specific primers

Primer name	Vector	Sequence 5'-3'
M13 S	pCR2.1	GTAAAACGACGGCCAG
M13 AS	pCR2.1	CAGGAAACAGCTATGAC
T7 S	pET151, pSECTag2B, pCDNA3.1, pDISPLAY2.0	TAATACGACTCACTATAGGG
BGH AS	pET151, pSECTag2B, pCDNA3.1, pDISPLAY2.0	TAGAAGGCACAGTCGAGG

Table 3.6: RT-PCR primers

Primer name	Sequence 5'-3'
Homology based primers	
Zebrafish *kitla* S1	TGCTGTACATCACAGTTGCTGCCT
Zebrafish *kitla* S2	AAAGAAGTGAGTGGCATGTGCTGGGT
Zebrafish *kitla* AS1	TTGCGCTGATGTTGTCGACGCTGTCGTAGT
Zebrafish *kitla* AS2	ACTGCGATTGCAAACGGGATGGTGAGGA
Zebrafish *kita* S1	ACCATCGTGTTCGACATTGTTGCGGCAGAA
Zebrafish *kita* S2	GTCGGAACTTTGGATGCCGCTACGGTTAAA
Zebrafish *kita* S3	CTGACGGAGCCCATTACTCAGGTGAGGACG
Zebrafish *kita* AS1	GTTCTCGCTGATAGAGAAGACTGTGTACACGAT
Zebrafish *kita* AS2	GGAGGACAGGATTCCCAGTACAGAGGGCTTCA
Zebrafish *kita* AS3	GTGGACTCTAAATTCTATAAGATGATCAAG
Zebrafish GCSFR S	AGCCTTCCAAGCCCTACGAGATCTC
Zebrafish GCSFR AS	GGCTGTGTGGACTCAGAGCGCA
RACE PCR primers	
Goldfish *kitla* 3' race	TGCCAATTTGTCAAGAGATGTCGAGGACTGCGAGC
Goldfish *kitla* nested 3' race	GCAGAACAGTCTTATGTCGCTCCTCACCATCCCGT
Goldfish *kitla* 5' race	ATTGGAGGATATGTTCCCAAACTTCTGCGCCAGG
Goldfish *kitla* nested 5' race	CGCAGTCCTCGACATCTCTTGACAAATTGGCAGT
Goldfish *kita* 5' race	AGGAGGCACGTCTGGAACGAGGCGAACA
Goldfish *kita* nested 5' race	TGTTGGGCAGGGACTGTCCGTCACATTT
Goldfish GCSFR 5' AS1	TCCAGCCATTGGCACTGAAATCTTCT
Goldfish GCSFR 5' AS2	AGCTGCCAGTTGGAGCCTTCATAAGT
Goldfish GCSFR 3' S1	TGGACCGACAGTGACTCATCGCAGG
Goldfish GCSFR 3' S2	CTGGCAGAGAACGCGCCATTCAAG
RT-PCR primers	
Zebrafish myeloperoxidase (AF349034) S	AGCCACTGCAGATGCTGACATAGA
Zebrafish myeloperoxidase (AF349034) AS	ATGTGGGATGTGAAGGCAAACAGC
Goldfish CSF-1R S	AGCCTCATCCAGACCCACAAA
Goldfish CSF-1R AS	GCGCCAGACCGAAATCACAGA
Goldfish β-actin S	CGAGCTGCGTGTTGCCCCTGAG
Goldfish β-actin AS	CGGCCGTGGTGGTGAAGCTGTTAG
Southern blot primers	
Goldfish GCSFR S	TCACACTCCTGCCACAAAGGTACT
Goldfish GCSFR AS	AAGTTCTTCACACACAGCACAAGC

Table 3.7: Quantitative PCR primers for goldfish genes

Primer name	Sequence 5'-3'
CEBPα S	ATCAAACAAGAGCCTCGGGAGGAA
CEBPα AS	TGGATTTCCCTCGATCGCCAATCT
cJun S	CGGCGATCCGGTTCCT
cJun AS	CCTCTCTCCCCCATCGACAT
cMyb S	GGGCTTACGGATGCATTAAAGA
cMyb AS	GAGCAGGGATGCCTTCCA
EF1α S	CCGTTGAGATGCACCATGAGT
EF1α AS	TTGACAGACACGTTCTTCACGTT
Egr1 S	TATCCTAACCGGCCAAGCAAGACA
Egr1 AS	TCTGCCGCATGTGGATCTTAGTGT
GATA1 S	GTCTGTTCTGGCCGTTCATTTT
GATA1 AS	ACCAGAGGCACGTGAATGC
GATA2 S	CACCATCCCATCCCAACCTA
GATA2 AS	GCTTTTGCATTTGGGTGTGA
GATA3 S	GTGTCCCCTGACCCCTCAAT
GATA3 AS	CGACTATGAGCAGAATCCAGCTT
GCSFR S	TCTTTTGGAATGAGAGGGACAAA
GCSFR AS	ACTTCCTCCCATCGTATGAACAG
KITA S	CAACTCATGTTCGCCTCGTT
KITA AS	CAGAGGCTGACCCAGTGTGA
KITLA S	TGGCTTGGAGGATTCAATGC
KITLA AS	TGGCCGTAAGCCACATCTC
Lmo2 S	TGCGTGTCCGTGACAAAGTC
Lmo2 AS	AAAAATGTCCTGCTCACACACAA
MafB S	CCAACATCAACACCAACAATACG
MafB AS	GACCCGGGCGAGATAGGA
Pax5 S	ATGAGACGGGCAGCATCAG
Pax5 AS	GATCTCCCAGGCAAACATGGT
Prominin S	TGCCTCTAATTGGCCTGTTCTT
Prominin AS	GGCCCCCGCAGTTACC
PU.1 S	TCGCCTCCTGTTGTTGATGTAA
PU.1 AS	CAGTCGCAGTCCTCCGTTAGA
Runx1 S	TCAAGGTAGTTGCCCTTGGTGATA
Runx1 AS	TTGAGGAGGGTTTGTGAAGACGGT
TNFα-2 S	TCATTCCTTACGACGGCATTT
TNFα-2 AS	CAGTCACGTCAGCCTTGCAG

Table 3.8: Recombinant protein expression primers

Primer name	Sequence 5'-3'
CSF-1 S	ACACACACATAACAGCCCACAAAGCC
CSF-1 AS	GGTTCAGTGGTGGATTCTGGGTA
pSECTag2B *kitla* S	AAGCTTTCCAGTGAAATAGGAAATCCCATTACA
pSECTag2B *kitla* AS	CTCGAGCCACAACTTTCGGAAGGAATGCCCC
Prominin S	CACCAGCAGTACAAACACTTCAGAGACT
Prominin AS	TTAAACATCCCGGCTGATTTGGCTTGA
Overlap Extension	
sCSF-1R SmaI pDISPLAY S	CCCGGGCAGGGTTGGTCTGAGCCGCGGATCAGA
O.Ext sCSF-1R+lpFcRyL AS	ATAACACAATCCACCCTCATGAAACTCACGCTGAAT
O.Ext sCSF-1R+lpFcRyL S	ATTCAGCGTGAGTTTCATGAGGGTGGATTGTGTTAT
lpFcRyL SalI pDISPLAY AS	GTCGACTTATGCCAAAGGTTCCTTCTTCACATGGAG

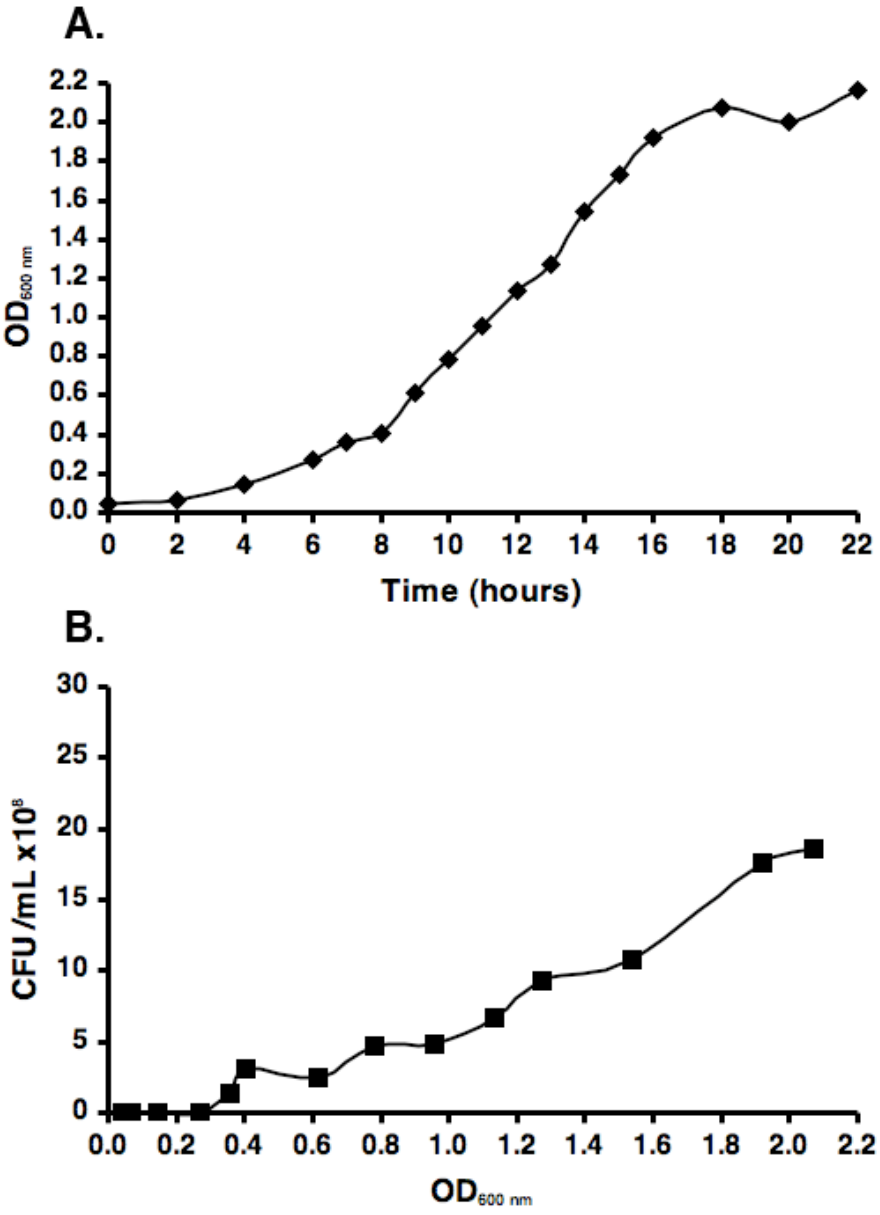


Figure 3.1: Growth curve of *Aeromonas salmonicida* A449.
Aeromonas salmonicida was grown for 22 hours at 18°C with shaking at 250 rpm. Samples were taken every 1-2 hrs to measure (A) optical density (OD) and (B) colony forming units (CFUs).

3.22 References

[1] **Wang R, Neumann NF, Shen Q, Belosevic M.** Establishment and characterization of a macrophage cell line from the goldfish. Fish Shellfish Immunol. 1995;5329-46.

[2] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[3] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[4] **Cortes HD, Montgomery BC, Verheijen K, Garcia-Garcia E, Stafford JL.** Examination of the stimulatory signaling potential of a channel catfish leukocyte immune-type receptor and associated adaptor. Dev Comp Immunol. 2011.

[5] **Bienek D, Belosevic, M.** Comparative assessment of growth of *Trypanosoma danilewskyi* Laveran and Mesnil 1904 in medium containing fish or mammalian serum. Journal of Fish Diseases. 1997;209.

[6] **Barreda DR, Hanington PC, Walsh CK, Wong P, Belosevic M.** Differentially expressed genes that encode potential markers of goldfish macrophage development in vitro. Dev Comp Immunol. 2004;28(7-8):727-46.

[7] **Southern E.** Southern blotting. Nat Protoc. 2006;1(2):518-25.

[8] **Bienek A.** Biology of fish trypanosomes. Edmonton: University of Alberta, 2001.

[9] **Zhang Z, Niu C, Storset A, Bogwald J, Dalmo RA.** Comparison of *Aeromonas salmonicida* resistant and susceptible salmon families: A high immune response is beneficial for the survival against *Aeromonas salmonicida* challenge. Fish Shellfish Immunol. 2011.

[10] **Hanington PC, Belosevic M.** Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. Dev Comp Immunol. 2007;31(8):817-29.

[11] **Heckman KL, Pease LR.** Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc. 2007;2(4):924-32.

[12] **Mewes J, Verheijen K, Montgomery BC, Stafford JL.** Stimulatory catfish leukocyte immune-type receptors (IpLITRs) demonstrate a unique ability to associate with adaptor signaling proteins and participate in the formation of homo- and heterodimers. Mol Immunol. 2009;47(2-3):318-31.

[13] **Barreda DR, Hanington PC, Stafford JL, Belosevic M.** A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[14] **Cubellis MV, Wun TC, Blasi F.** Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. EMBO J. 1990;9(4):1079-85.

[15] **Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR.** Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982;126(1):131-8.

Chapter 4: Molecular and functional analysis of goldfish kita and kit ligand a[1]

4.1 Introduction

Kit ligand, also known as stem cell factor (SCF), mast cell growth factor, and steel factor in mammalian systems, and its type III tyrosine kinase receptor c-Kit are involved in hematopoiesis [1-3], spermatogenesis [4, 5], and development of melanocytes [5-7] and mast cells [8-11]. Early studies reported that c-Kit was encoded by the *White* locus (*W*) in mice as the normal homologue of the Hardy-Zuckerman 4 feline sarcoma virus [12, 13]. c-Kit is structurally similar to the type III tyrosine kinase receptors, such as colony stimulating factor-1 receptor (CSF-1R) and platelet-derived growth factor receptor (PDGFR) [14]. These receptors are characterized by an extracellular domain consisting of five immunoglobulin-like domains and by an insertion of ~70 to 100 amino acids in the middle of the intracellular tyrosine kinase signaling domain [15]. SCF was later identified by various groups [16-18] as short-chain four-helix bundle [19] encoded by the *Steel* locus in the mouse [12]. SCF is extensively glycosylated with both N- and O-linked sugars that make up approximately 30% of its molecular weight [20]. Two of these monomers non-covalently associate in a "head-to head" manner to form a homodimer that binds to the second and third immunoglobulin domains of the Kit receptor with a high affinity [20-25]. Binding of SCF to c-Kit results in receptor dimerization and conformational changes in the D4 and D5 domains of c-Kit resulting in autophosphorylation of the intracellular

[1] A version of this chapter has been published. Katzenback and Belosevic, 2009. *Developmental and Comparative Immunology* 33:1165-1175.

tyrosine kinase domains and downstream signaling [26]. Mice with mutations in the *White* or *Steel* loci exhibit hypopigmentation, mast cell deficiency, macrocytic anemia, and sterility, while complete loss of either of these genes is lethal [12, 27].

In lower vertebrates such as the zebrafish, genomic duplication has resulted in the expression of kit ligand A (*kitla*) and B (*kitlb*) as well as kit receptor A (*kita*) and B (*kitb*) [28-30]. However, the function seems to be partitioned to *kita* and *kitla* with regards to melanocyte survival, migration and differentiation [30-34]. While studies in zebrafish have shown the importance of *kita* and *kitla* in melanocyte development, they did not observe obvious defects in hematopoiesis, suggesting that *kita* and *kitla* were not essential to hematopoiesis in zebrafish. These studies on *kita* and *kitla* are in contrast to those performed in mammalian systems where signaling of SCF through c-Kit promotes the survival, proliferation and differentiation of progenitor cells involved in hematopoiesis. While zebrafish studies suggest that *kita* and *kitla* may not have an essential role in hematopoiesis, they do provide some evidence for their involvement in myeloid development as significant differences in proportions of myeloid cells, particularly promyelocytes, exist between wild type and kit (*sparse*) mutant zebrafish [28].

This chapter describes the identification and molecular and functional characterization of goldfish *kita* and *kitla*. To date, the role of *kita* and *kitla* during myeloid development in teleosts has not been fully examined, and further characterization of this receptor-ligand pair is required before its contribution to myelopoiesis can be defined.

The main objectives of this chapter were to (1) identify goldfish *kita* and *kitla* transcripts, (2) assess the distribution of *kita* and *kitla* mRNA levels in

goldfish tissues, cell populations and in response to pathogens, (3) generate a recombinant KITLA protein (rgKITLA) and determine if this recombinant protein was glycosylated and formed homodimers, (4) functionally characterize rgKITLA in terms of its role in promoting chemotaxis of PKMs, maintaining progenitor cell survival, driving differentiation of progenitor cells, and in the proliferation of progenitor cells.

4.2 Results

4.2.1 Phylogenetic analysis of goldfish *kita* and *kitla*

Phylogenetic analysis of the partial goldfish *kita* sequence placed it amongst other Kit receptors in the type III tyrosine kinase family (Fig. 4.1) and was most similar to *Danio rerio kita* (AAI62502). Using the SMART program (ExPASy Proteomics server) and the conserved domains program (NCBI) the protein is predicted to have four extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain containing an active site, an ATP binding site, a substrate binding site, and an activation loop (A-loop). The presence of the immunoglobulin domains, the tyrosine kinase intracellular signaling domain and high similarity to *Danio rerio kita* suggests that this transcript encodes for the goldfish KITA type III tyrosine kinase receptor.

Similar to that observed for goldfish KITA, phylogenetic analysis of goldfish KITLA placed it with other Kit ligands (Fig. 4.2), and was most similar to *Danio rerio* KITLA (AAX76926) with 85% amino acid identity, *Gasterostus aculeatus aculeatus* KITL (ABW91106) with 50%, *Danio rerio* KITLB (ZZX76927) with 31% identify, and only 28% amino acid identity with *Xenopus laevis* KITL

(NP_001080125). Additionally, analysis of the protein sequence using the conserved domains program (NCBI) identified the predicted protein to contain a SCF superfamily domain. Based on these analyses I believe this transcript to encode for goldfish KITLA. The predicted goldfish KITLA protein was 272 amino acids with a secretion signal peptide cleavage site between amino acid 22 and 23 (Fig. 4.3, arrow). The protein is approximately 28 kDa with an isoelectric point of 5.32. The predicted protein possessed four cysteine residues that are important in the formation of the disulfide bonds for proper folding and function of KITLA (Fig. 4.3, diamonds). Like that of mammalian SCF proteins, goldfish KITLA is predicted to be glycosylated (ExPASy Proteomics server) with two potential N-linked glycosylations (Fig. 4.3, triangles) and fourteen potential O-linked glycosylations (Fig. 4.3, circles). A trans-membrane region was also predicted to span between amino acids 207-224 (Fig. 4.3, bar) and suggests a membrane bound form of goldfish KITLA. The high similarity between goldfish KITLA and zebrafish KITLA suggests the presence of exon 6 which encodes for a proteolytic cleavage site which would release membrane bound KITLA (mKITLA) to a soluble KITLA (sKITLA) form [30].

4.2.2 Expressions of goldfish *kita* and *kitla* in tissues, PKM cultures, and sorted PKM subpopulations

To determine the distribution and mRNA levels of goldfish *kita* and *kitla* in tissues, the blood, heart, brain, gill, intestine, kidney and spleen of four individual fish were harvested and used for quantitative PCR. The mRNA levels of *kita* and *kitla* in the tissues ranged between ~1.5 and 2 fold increase compared to the mRNA levels in the heart reference tissue. Both goldfish *kita* and *kitla* were

ubiquitously expressed in the tissues examined, with highest expression observed in the kidney, spleen and brain (Fig. 4.4A and B).

To examine the role of goldfish *kita* and *kitla* in monopoiesis, their gene expression was examined in PKM cultures from three fish in complete NMGFL-15 medium over 8 days of cultivation. Two million cells were seeded per well, and at day 0, day 2, day 4, and day 8 of cultivation, mRNA levels of *kita* and *kitla* of PKM cells were assessed. Due to the variability in gene expression of PKM cultures at day 0, the mRNA levels of *kita* and *kitla* at day zero were set to a value of one. Subsequent days were expressed as a fold change in mRNA levels. Both *kita* and *kitla* mRNA levels decreased over the eight days of cultivation (Fig. 4.5A). However, *kita* expression decreased to a greater extent than *kitla* at all time points observed with the lowest expression of *kita* and *kitla* observed on day 8 of cultivation (Fig. 4.5A). These data initially suggested that *kita* and *kitla* may have a role in progenitor cell biology, as *kita* and *kitla* mRNA levels decreased as mature macrophages appear in culture.

To determine if *kita* or *kitla* mRNA levels could be upregulated in macrophages, day 8 PKMs from four fish were pooled and treated with LPS or heat-killed *A. salmonicida*. Interestingly, *kita* and *kitla* mRNA levels increased in macrophages treated with either LPS or heat-killed *A. salmonicida* A449 (Fig. 4.5B). Goldfish *TNFα-2* expression was used as a positive control as a measure of cell activation. In the case of LPS stimulation, *TNFα-2* expression was slightly higher than that of non-treated cells, whereas *A. salmonicida* stimulation increased *TNFα-2* expression ~3.5 fold (Fig. 4.5B). Goldfish *kita* expression was ~2-2.5 fold higher in activated cells, regardless of whether they were stimulated

with LPS or *A. salmonicida*, whereas *kitla* expression was ~1.5 and ~2.0 fold higher after stimulation with LPS and *A. salmonicida*, respectively (Fig. 4.5B).

To further elucidate the cell populations responsible for the *kita* and *kitla* mRNA level changes in PKM cultures, progenitors, monocytes and macrophages from three fish were sorted using a FACSCalibur flow cytometer (Becton Dickinson) and *kita* and *kitla* mRNA levels analyzed using quantitative PCR. Progenitor cells were observed to have the highest mRNA levels of *kita* compared to monocytes and macrophages (Fig. 4.6A). However, progenitor cell *kita* mRNA levels were highly variable, most likely due to the varying sub-populations of cells that make up the progenitor cell pool as well as the varying developmental stages of the progenitor cells within the pool of progenitors. Nevertheless, the decreasing expression of *kita* as progenitors develop into monocytes and macrophages is in accordance with the previous PKM culture observations and suggests a role for KITA in progenitor cell signaling. Both progenitor cells and monocytes expressed the highest mRNA levels of *kitla* compared to macrophages (Fig. 4.6B), and these data are consistent with the higher levels of *kitla* mRNA compared to *kita* mRNA levels during cultivation (Fig. 4.5A).

4.2.3 Recombinant goldfish KITLA expression and purification

The extracellular portion of *kitla* was cloned into the pSECTag2B plasmid containing an N-terminal secretion signal and a C-terminal myc and six histidine tag. The construct was transfected into HEK 293T cells to produce a recombinant goldfish KITLA (rgKITLA). Following collection of HEK 293T cell culture

supernatants, supernatants were incubated with nickel beads (NiNTA-agarose beads, Qiagen), and beads were washed with elution buffer with increasing amounts of imidazole to remove recombinant protein bound by their 6x His tag. Elution fractions were run on an SDS-PAGE gel, transferred to a nitrocellulose membrane and blotted with an anti-His antibody. The predicted molecular weight of the recombinant protein, excluding glycosylation, was ~25 kDa. However, the recombinant KITLA was observed to be ~40 kDa and had a smeared appearance characteristic of glycosylated proteins suggesting that the rgKITLA protein is also highly glycosylated as it is in mammals (Fig. 4.7). Mass spectrometry analysis of the ~40 kDa band identified this protein as most similar to zebrafish KITLA when compared against the NCBI database, and matching of the generated peptides with the goldfish KITLA sequence confirmed the identity of the recombinant protein.

4.2.4 Deglycosylation of rgKITLA

To determine if rgKITLA was glycosylated, rgKITLA was incubated with PNGase F, which is known to cleave N-linked glycosylations. Following incubation with or without PNGase F, samples were subjected to treatment with or without β2-mercaptoethanol to break disulphide bonds, and run on a reducing gel. Following blotting with an anti-myc-HRP antibody, bound antibodies were visualized with a chemilluminescent detection system. Samples untreated with beta-2 mercaptoethanol or PNGase F migrated to around 40 kDa, as expected (Fig. 4.8A). However, when treated with PNGase F, followed by no treatment or treatment with beta-2-mercaptoethanol, there was a decrease in the molecular

weight in which the rgKITLA migrated, ~ 34 kDa, and the band was more condensed in appearance compared to the non-PNGase F treated samples (Fig. 4.8A). This suggested that rgKITLA possesses N-linked glycosylations as predicted by *in silico* analysis. It is likely that the additional size of rgKITLA is the result of the predicted O-linked glycosylations.

4.2.5 Cross-linking of rgKITLA

The dimerization of SCF is important for signaling through KIT in mammalian systems, and is required to induce dimerization of the receptor that leads to autophosphorylation. To determine whether the recombinant goldfish KITLA could dimerize, a cross-linking experiment was performed. Recombinant goldfish KITLA was incubated in the presence or absence of Bis (sulfosuccinimidyl) suberate (BS^3), a cross-linking agent that forms non-cleavable bonds between the amine groups. In the absence of BS^3, rgKITLA migrated to around 40 kDa (Fig. 4.8B, left blot, left lane). However, in the presence of BS^3, a band of approximately 70-80 kDa and an additional band of approximately 150-160 kDa was observed (Fig. 4.8B, left blot, right lane). The band around 70-80 kDa is believed to represent a dimerization of rgKITLA, while the 150-10 kDa band is believed to possibly represent a tetramer of rgKITLA. The interaction of rgKITLA into dimers and tetramers is specific, as the addition of bovine serum albumin (BSA) to rgKITLA in the presence of BS^3 did not interfere with the banding pattern observed in the sample of rgKITLA with BS^3 (Fig. 4.8B, right blot). These data show that recombinant goldfish KITLA forms dimers and may form novel tetramers that could modulate signaling through the KITA receptor.

4.2.6 Recombinant goldfish KITLA induced a chemotactic response of cells from early and mature PKM cultures

To investigate the chemotactic effect of recombinant KITLA, early macrophage cultures (day 2) consisting of mainly progenitor cells were chosen as they demonstrated the highest level of *kita* mRNA expression. For the chemotaxis assay, duplicate filters were used for each treatment, and two fish were used per experiment. Duplicate experiments were performed. In the presence of recombinant goldfish leukemia inhibitory factor (rgLIF), leukocytes from a day 2 PKM culture migrated towards the source and this migration was statistically significant compared to the negative control of medium alone ($P<0.05$; one-way ANOVA) (Fig. 4.9A). Recombinant goldfish LIF has been shown in our lab to be chemoattractive (unpublished data). When varying concentrations of recombinant goldfish KITLA were placed in the lower chemotaxis chamber, leukocytes migrated towards the recombinant cytokine in a dose-dependent manner. The highest level of migration was observed when 100 ng/mL of rgKITLA was placed in the bottom chamber, ~225 cells/20 fields of view (Fig. 4.9A). However, chemotaxis did not occur at lower concentrations of recombinant KITLA (>10 ng/mL), or at the 1000 ng/mL concentration of recombinant KITLA (Fig. 4.9A). Interestingly, a significant chemotactic response was observed towards a very low concentration of rgKITLA (0.001 ng/mL) (Fig. 4.9A). Furthermore, this chemotactic response was not due to random migration as the chemokinesis control was not significantly different from the medium alone control.

To further investigate the chemoattractive nature of recombinant KITLA on mature PKM cultures, a chemotaxis assay was performed using days 6-9 PKM cultures consisting of mainly macrophages. For the chemotaxis assay, duplicate filters were used for each treatment, and two fish were used per experiment. Duplicate experiments were performed. Similarly, mature macrophages migrated towards rgLIF and this migration was statistically significant from the medium alone control ($P<0.05$). In the presence of varying concentrations of recombinant KITLA, macrophages exhibited a dose-dependent chemotactic response towards higher concentrations of recombinant KITLA, however this response was abolished when concentration of the cytokine was greater than 100 ng/mL (Fig. 4.9B). Again, the chemotactic response was determined to be due to directional migration rather than random migration as indicated by non-significant chemokinetic response results compared to the medium alone negative control (Fig. 4.9B).

4.2.7 Recombinant goldfish KITLA induced proliferation of progenitor cells from early PKM cultures

To assess the ability of rgKITLA to promote proliferation of progenitor cells, day 2 PKMs from two fish were seeded in duplicate wells and treatments consisted of medium alone, cell-conditioned medium (CCM) from prior PKM cultures, or a range of rgKITLA concentrations. Plates were developed at day 0, 2, 4 and 6 days post treatment. This experiment was performed in duplicate. Treatment of early (day 2) PKM cultures with 0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, or 1000 ng/mL of recombinant KITLA induced a dose-dependent proliferative response in early progenitor cells, with the highest

proliferation observed when cells were treated with 0.01 ng/mL or 0.1 ng/mL of rgKITLA. Although rgKITLA did not induce higher proliferation on days 2 and 4 post treatment compared to cell conditioned medium (CCM, positive control), the proliferative responses of cells treated with 0.01 ng/mL or 0.1 ng/mL of rgKITLA were similar to those induced by CCM on day 6 post treatment, and a modest proliferative response was observed in cells treated with 1 ng/mL or 10 ng/mL of rgKITLA (Fig. 4.10). Interestingly cells treated with higher concentrations of recombinant KITLA (100 ng/mL or 1000 ng/mL) did not to proliferate (Fig. 4.10) which may be the result of rapid ligand-receptor mediated internalization and degradation that has been documented for c-KIT in mammals [10] or receptor desensitization [35] at high ligand concentrations. In these experiments, the values for cells in serum-free NMGFL-15 medium were subtracted from all experimental groups to control for proliferation due to the production of endogenous growth factors.

4.2.8 Recombinant goldfish KITLA promoted survival of progenitor cells *in vitro*

To assess the ability of recombinant goldfish KITLA to induce differentiation of progenitor cells into monocytes or macrophages, day 2 PKM cultures from three fish were seeded at 1 x 10^6 cells/well in a 6 well plate treated with either serum-free NMGFL-15 only (negative control) CCM (positive control) or 0.1 ng/mL, 1 ng/mL, 10 g/mL or 100 ng/mL of recombinant KITLA in serum-free NMGFL-15 medium and incubated for 8 days. Starting on day 0 and every 2 days thereafter, flow cytometry analysis of the cell populations was performed.

Two observations could be made using flow cytometry analysis of the PKM cultures: progenitor cell differentiation and progenitor cell survival. While CCM induced the differentiation of progenitor cells into monocytes and macrophages, as we reported previously [36, 37], differentiation of progenitor cells into monocytes or macrophages was not observed in wells treated with rgKITLA or medium control groups (data not shown). To assess the ability of rgKITLA to promote survival of progenitor cells *in vitro*, a numerical assessment of the percentage of progenitor cells in each culture during the observation period was determined by enumerating cells found in the R1-gate of the flow cytometer (= progenitor cell pool) [36, 37]. Survival was measured as the percentage of progenitor cells remaining at the various time points compared to the number of progenitor cells present at day 0. Cells treated with CCM consistently had lower numbers of progenitor cells compared to cells treated with medium alone at all time points (Fig. 4.11). This is consistent with the previous published results of a decrease in a progenitor subpopulation as macrophage development progresses in the presence of CCM or complete medium [38]. In contrast, a statistically significant percent of progenitor cells remained following treatment with 10 ng/mL or 100 ng/mL of rgKITLA on day 2 of cultivation compared to that of medium control ($P<0.05$). The percentages of progenitor cell remaining in cultures treated with 1 ng/mL and 0.1 ng/mL of rgKITLA were significant compared to those of time matched medium control ($P = 0.06$ and $P = 0.07$, respectively). On day 4 of cultivation, cells in all four groups treated with rgKITLA had significantly higher percent of progenitor cells remaining compared to the time matched medium control ($P<0.05$) (Fig. 4.11), and by day 6 of cultivation, only the progenitor cells treated with 100 ng/mL of recombinant KITLA had a higher percentage of

progenitor cells remaining than that of medium alone control (P = 0.052). By day 8 of cultivation there were no significant differences in percent of progenitor cells in all experimental groups when compared to time matched medium control (Fig. 4.11).

4.2.9 Recombinant goldfish KITLA does not induce a nitric oxide response in mature macrophages

Due to the migratory response of macrophages to rgKITLA observed during the chemotaxis assay, an assessment of whether macrophages could become activated in response to rgKITLA was assessed using a nitric oxide assay. Day 6-8 PKMs from four fish were incubated with medium alone (negative control), transferrin macrophage activating peptide (TMAP) or varying concentrations of rgKITLA for 48 hours before the measurement of nitrite production. TMAP produced a significant nitric oxide response in macrophages compared to the medium control; however, rgKITLA, at any concentration, did not produce a nitric oxide response in macrophages (Fig. 4.12).

4.2.10 Production of a polyclonal antibody to rgKITLA

Prior to immunization, approximately 3 mLs of blood was obtained as the pre-bleed. A New Zealand white rabbit was immunized with 125 μg of rgKITLA in Freunds complete adjuvant, followed by three boosters of 100 μg of rgKITLA in Freunds incomplete adjuvant. Rabbits were bled and rabbit IgG purified from the serum using affinity purification. The protein concentration was determined using a BCA assay (Pierce) and determined to be 256 μg/mL. To determine the ability

of the anti-rgKITLA antibody to recognize rgKITLA, rgKITLA was run on a native or reducing gel, and individual lanes cut into strips. Individual strips were incubated with increasing dilutions of the anti-rgKITLA antibody. The anti-rgKITLA antibody recognized both native (Fig. 4.13A) and reduced rgKITLA (Fig. 4.13B) in a western blot. The anti-rgKITLA antibody recognized the native rgKITLA up to a 1:40,000 antibody dilution (Fig. 4.13A) and recognized the reduced form of rgKITLA up to a 1:80,000 antibody dilution (Fig. 4.13B). The non-immune IgG from the pre-bleed did not recognize the rgKITLA protein at the highest antibody dilution tested (Fig. 4.13A and B). Additionally, the anti-rgKITLA antibody was able to recognize deglycosylated rgKITLA (Fig. 4.8A). Therefore, the anti-rgKITLA antibody can recognize rgKITLA in both native and reduced forms, in the presence or absence of glycosylations.

To determine if the anti-rgKITLA antibody could be used in immunoprecipitation studies, the ability of anti-rgKITLA to pull down rgKITLA from solution was assessed. Two μg of rgKITLA was incubated with anti-recombinant *Trypanosoma carassii* calreticulin (anti-CRT) or varying concentrations (0 μg, 1 μg, 2 μg, 4 μg and 8 μg) of anti-rgKITLA for 2 hours. To the protein-antibody mixture, Protein G beads were added and incubated for overnight. Following incubation, the supernatant was removed from the beads (S) and the beads were washed to remove any unbound protein, SDS-PAGE loading buffer added to the beads and boiled to dissociate antibody-protein complexes from the beads (B). The supernatant was run on a reducing gel along with a well containing 2 μg of rgKITLA as a loading control. Following transfer to a nitrocellulose membrane, blots were probed with an anti-myc HRP antibody that recognizes the myc epitope on the C-terminus of the rgKITLA protein (Fig. 4.14A and B). The anti-

CRT antibody did not bind rgKITLA as rgKITLA was only detected in the supernatant and not the bead fraction (Fig. 4.14A). However, when beads were pre-incubated with 8 μg of anti-rgKITLA, a large fraction of the total rgKITLA was detected in the bead fraction, suggesting that anti-rgKITLA bound rgKITLA (Fig. 4.14A). The binding of rgKITLA by anti-rgKITLA decreased in a dose-dependent manner with decreasing concentrations of anti-rgKITLA (Fig. 4.14A and B). Accordingly, there was an increase in the amount of rgKITLA detected in the supernatants with decreasing amounts of anti-rgKITLA antibody (Fig. 4.14A and B). Furthermore, rgKITLA does not bind to the beads in a non-specific manner. rgKITLA was only detected in the supernatant fraction in the sample of beads incubated with 2 μg of rgKITLA in the absence of anti-rgKITLA. The anti-rgKITLA antibody did not detect recombinant CRT in a subsequent western blot.

4.2.11 Attempts to detect native KITLA and KITA

Attempts to detect native KITLA in PKMs was performed using immunoprecipitation with the anti-rgKITLA antibody. Day 2 PKMs from two fish were collected and their surface proteins biotinylated. Cells were washed to remove excess labeling reagent. Following washing steps, cells were lysed in a lysis buffer containing Triton X to solubilize membrane proteins, and the combined lysates cleared of membranes. PKM cell lysates were incubated without antibody, with anti-CRT antibody, or with anti-rgKITLA antibody. Following overnight incubation, Protein G beads were added to the antibody-lysate mixture and incubated for an additional hour. The supernatant was aspirated, the beads were washed, and both fractions run on an SDS-PAGE gel

for all three treatments. However, in all treatments, a non-specific banding pattern was observed for the bead fraction. These observations suggest that the goldfish proteins are binding to the Protein G beads in a non-specific manner. Likely, the optimization of the binding, incubation and washing conditions will be required before the native molecule can be detected. Additionally, the failure of this experiment may be due to the low concentration of native KITLA expressed on cells. Alternatively, future experiments should focus on generating highly concentrated CCM from early PKM cultures and trying to detect the native KITLA from this preparation.

Lastly, an experiment was designed to detect native KITA on the surface of progenitor cells based on a protocol used in a porcine system to detect KITA on the surface of progenitor cells [39]. The experimental design consisted of incubating day 0 or day 2 PKMs with 0 ng, 100 ng or 1000 ng of rgKITLA for 30 minutes on ice in a FACS buffer containing 0.05% sodium azide to prevent receptor internalization. Cells were washed twice, and then incubated with an anti-His-PE conjugated antibody. Samples were then run on the FACS Calibur flow cytometer. However, a shift in fluorescence was not observed in any of the treatments for the day 0 or day 2 PKMs. I was likely unable to detect goldfish KIT positive cells due to the low numbers of KIT receptors on progenitor cells combined with the small population of cells expressing KIT receptor.

4.3 Discussion

In this chapter, I described the cloning and characterized goldfish *kita* and *kitla*. In addition, the production and characterization of an anti-rgKITLA antibody,

as well as attempts to isolate native goldfish KITLA or KITA, were described. Goldfish KITA has extracellular immunoglobulin domains and an intracellular tyrosine kinase domain, characteristic of tyrosine kinase receptor family members that include colony stimulating factor 1 receptor (CSF-1R), platelet derived growth factor receptor (PDGFR), and the F1 cytokine receptor (Flk2/Flt3) [1, 40]. Goldfish KITA is structurally very similar to zebrafish KITA with four immunoglobulin domains. While mammalian c-KIT has five extracellular immunoglobulin domains, as is characteristic of other type III tyrosine kinase receptors [40], it appears that all teleost KITAs identified to date possess four immunoglobulin domains.

Goldfish KITLA is structurally very similar to zebrafish KITLA with 85% amino acid identify, both are 272 amino acids in length, and have four conserved cysteine residues at identical positions to zebrafish KITLA. *In silico* analysis of the predicted protein structure identified a conserved SCF superfamily domain located at the N-terminus. Based on genomic analysis performed in the zebrafish and the high similarity between goldfish and zebrafish KITLA protein sequences, I suspect that goldfish KITLA may be expressed as a membrane bound form as well as a soluble form after proteolytic cleavage [30].

Both *kita* and *kitla* were ubiquitously expressed in the tissues examined, with highest expression in the kidney, spleen, and brain. Since the major hematopoietic organs of fish are the kidney and spleen, I would expect both *kita* and *kitla* to be expressed highly in these tissues if they play a role in myelopoiesis. In mammalian systems *c-KIT* mRNA is widely distributed and was reported to be present in embryonic brain, interstitial cells of Cajal, and renal tubules [41-45]. The possible role of c-KIT and SCF in maintaining differentiated

cell or tissue types has been proposed as mechanism for this broad c-KIT expression in higher vertebrates.

The role of c-KIT and SCF in hematopoiesis is well documented in mammals [1, 46, 47]. However, little is known regarding the function of KITA and KITLA in myelopoiesis of lower vertebrates. Our laboratory has a unique *in vitro* primary kidney macrophage culture system where developing macrophage subpopulations are present: progenitors, monocytes, and mature macrophages [36, 37, 48, 49]. To examine the role of KITA and KITLA in the context of the *in vitro* PKM culture system, the mRNA levels of *kita* and *kitla* were determined using quantitative PCR. I observed the highest *kita* and *kitla* mRNA levels in freshly isolated kidney leukocytes (day 0 of cultivation) that consistently decreased with duration of cultivation. While *kitla* mRNA levels decreased over time, they did not decrease as dramatically as *kita* mRNA levels. When cells from PKM cultures were sorted into progenitor, monocyte and macrophage subpopulations, the highest mRNA levels of *kita* were observed in progenitor cells, suggesting that these cells are primarily responsible for the gene expression of *kit* in PKM cultures. Furthermore, as progenitor cells mature into macrophages *in vitro*, the expression of *kita* decreases akin to that observed when the complete PKM cultures that were use for initial expression analysis. While monocytes (R3s) and mature macrophages (R2s) from our PKM cultures have been functionally characterized, relatively little is known about the progenitor cell (R1) subpopulation. The utilization of whole kidney to generate these cultures would suggest the presence of a multitude of progenitor cells of varying lineages [50, 51], including progenitor B-cells [52]. It has been demonstrated in mammalian systems that pro-B cells are c-KIT positive,

however, evidence of the presence or absence of KITA or KITB on teleost B-cell progenitors including those of goldfish has not been shown. Thus, it remains a possibility that the likely presence of progenitor B-cells in our PKM cultures may account in part for the expression of *kita* observed in the progenitor cell (R1) population.

Our results suggest that high *kita* mRNA levels may be related to the observed functional effect of the recombinant KITLA protein, specifically, progenitor cell maintenance (see below). Similarly, *kitla* expression was highest in progenitor cells and monocytes compared to mature macrophages. These results are consistent with the delayed down-regulation of *kitla* expression in PKM cultures as the presence of progenitors and monocytes in the cultures may account for the prolonged expression of *kitla*. The expression of *kitla* transcripts in both progenitors and monocytes provides additional evidence that fish mononuclear cells can produce their own growth factors as previously described [36, 53-55] and provides insight into the dynamic of our PKM culture system in terms of maintenance of a progenitor cell population and growth requirements of developing macrophages. The up-regulation of *kita* and *kitla* mRNA levels in macrophages exposed to LPS and *A. salmonicida* suggests a possible role for *kita* and *kitla* in mast cell activation at the inflammatory sites [11], or increased hematopoiesis in situations where enhanced cell mobilization and chemotactic responsiveness would improve host defense [56]. SCF has been shown to be chemoattractive to myeloid cells in mammals [57], which is similar to what I have documented for goldfish recombinant KITLA in this study. Similar to SCF, goldfish KITLA also induced proliferation and survival of progenitor cells [2, 57-59]. Interestingly, rgKITLA did not induce an immediate proliferative response in

cells from early PKM cultures, suggesting that KITLA may be synergizing with endogenous growth factors produced by our PKM cells [36, 53-55, 60].

This is the first report on the effects of goldfish recombinant KITLA on mononuclear progenitor cells of teleosts. Although previous studies performed in zebrafish suggest KITA and KITLA do not perform an essential role in hematopoiesis, this may be dependent on the developmental stage of the zebrafish. A study examining the requirement of c-KIT and SCF in mice embryos demonstrates a similar c-KIT-independent mechanism in the first wave of hematopoiesis with minimal defects in blood development. However, the second wave of hematopoiesis in the mouse embryo is c-KIT-dependent and the blocking of c-KIT with an antibody results in significant defects in myeloid and erythroid progenitor cell numbers [61]. An alternative explanation could be the presence of another receptor-ligand pair in lower vertebrates that control early signaling in hematopoiesis.

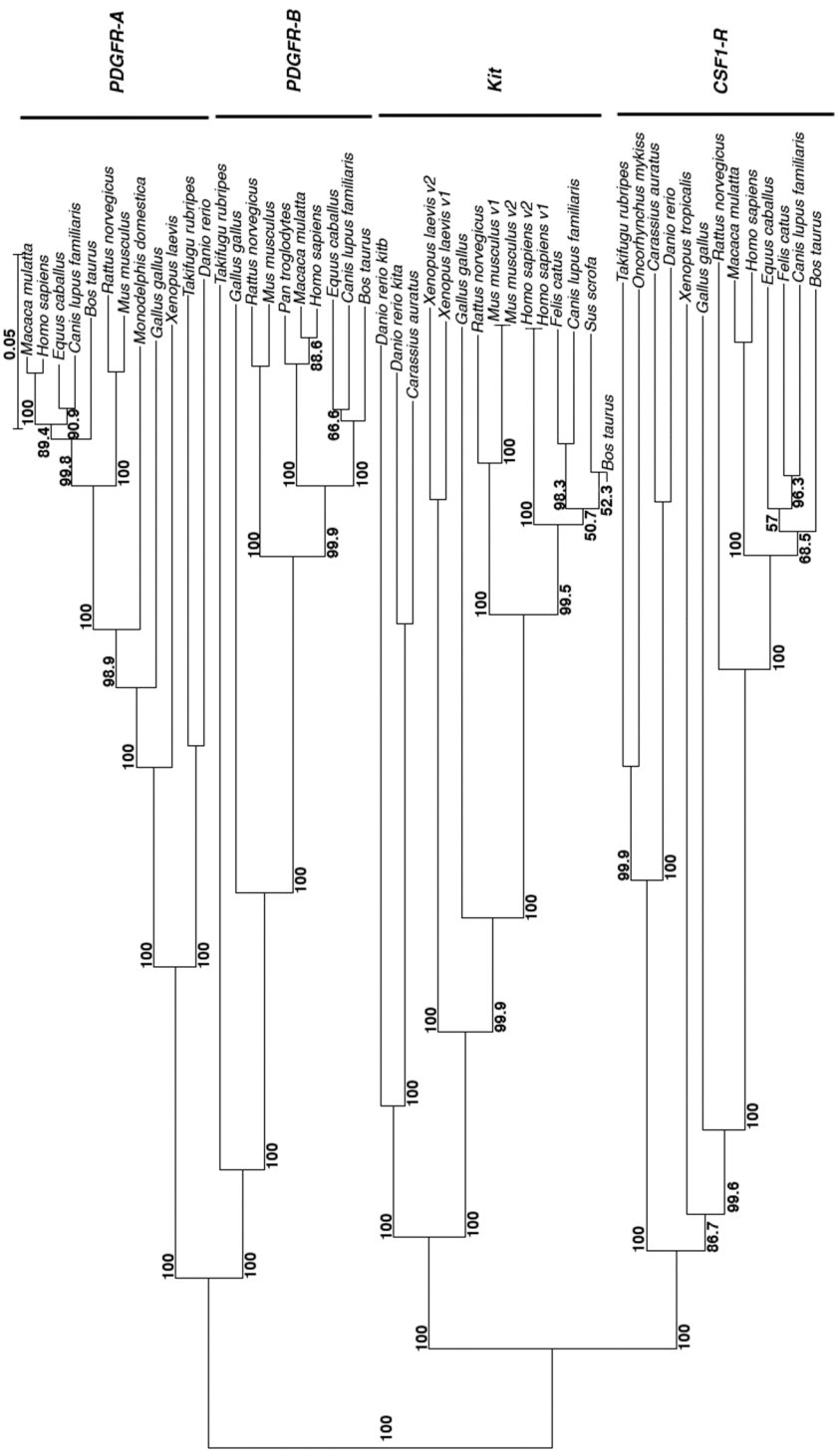


Figure 4.1: Phylogenetic analysis of goldfish *kita*.
Goldfish *kita* grouped closely with zebrafish *kita* amongst other kit receptors.The Kit receptors grouped closely with the other type III tyrosine kinase receptors CSF1-R, and PDGFR A and B. The phylogenetic tree was bootstrapped 10,000 times and expressed as a percentage.

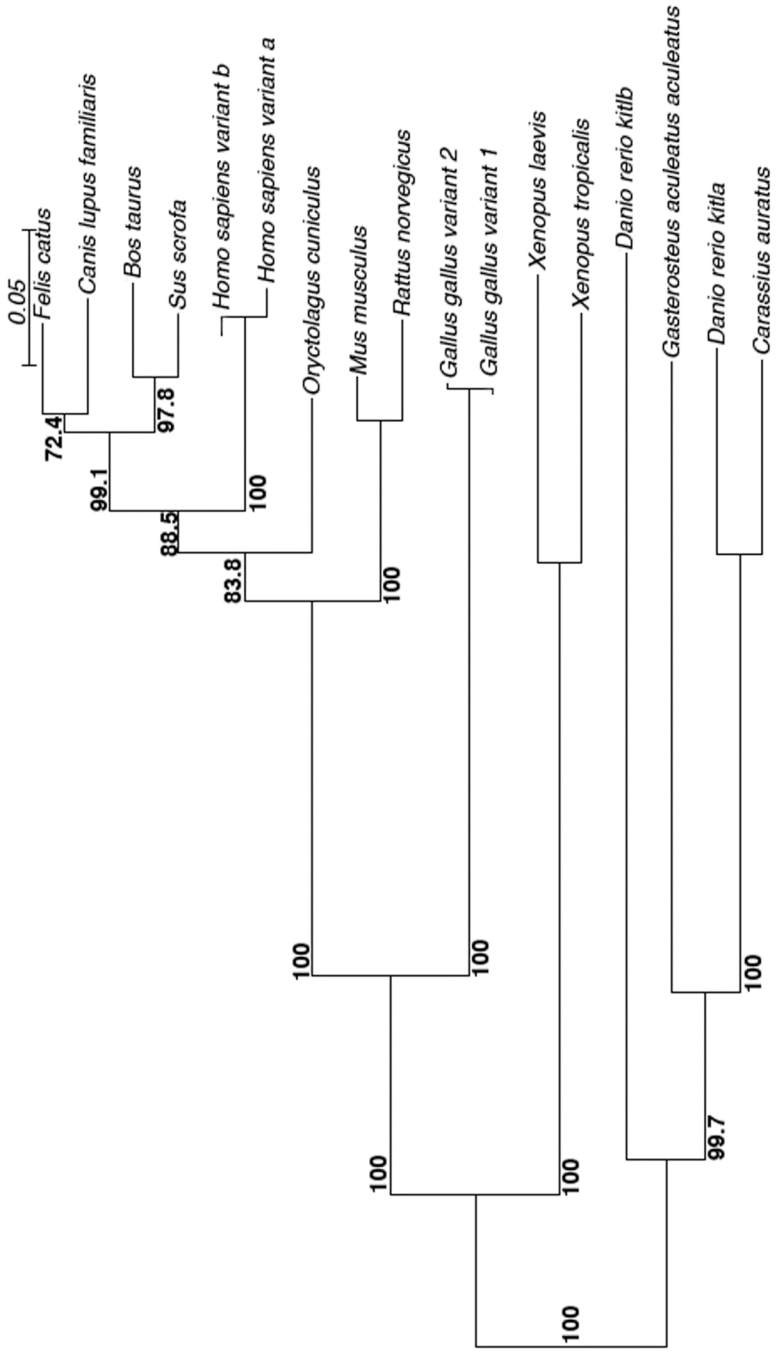


Figure 4.2: Phylogenetic analysis of goldfish kit ligand A (*kitla*).
Goldfish *kitla* grouped closely with zebrafish *kitla* and appear to be most closely related with other fish and frog *kit* ligands. The phylogenetic tree was bootstrapped 10,000 times and expressed as a percentage.

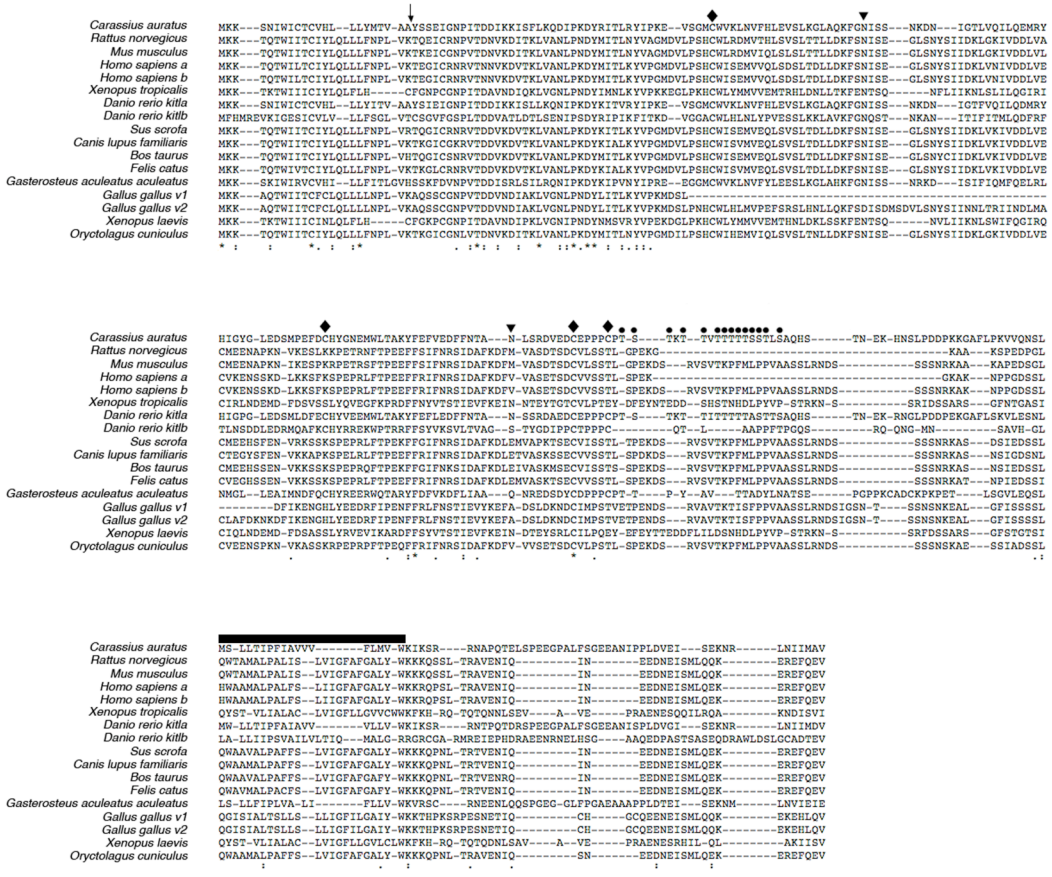


Figure 4.3: Amino acid alignment of KIT ligands.
Amino acids that are conserved in all sequences are denoted with a (*), with high identity (:), and with weak identity (.). The signal peptide cleavage site for goldfish KITLA is denoted with an arrow, conserved cysteine resides are denoted with a diamond, predicted O-glycosylations are denoted with a circle, predicted N-glycosylations are denoted with a triangle, and the predicted transmembrane region is donated with a solid bar.

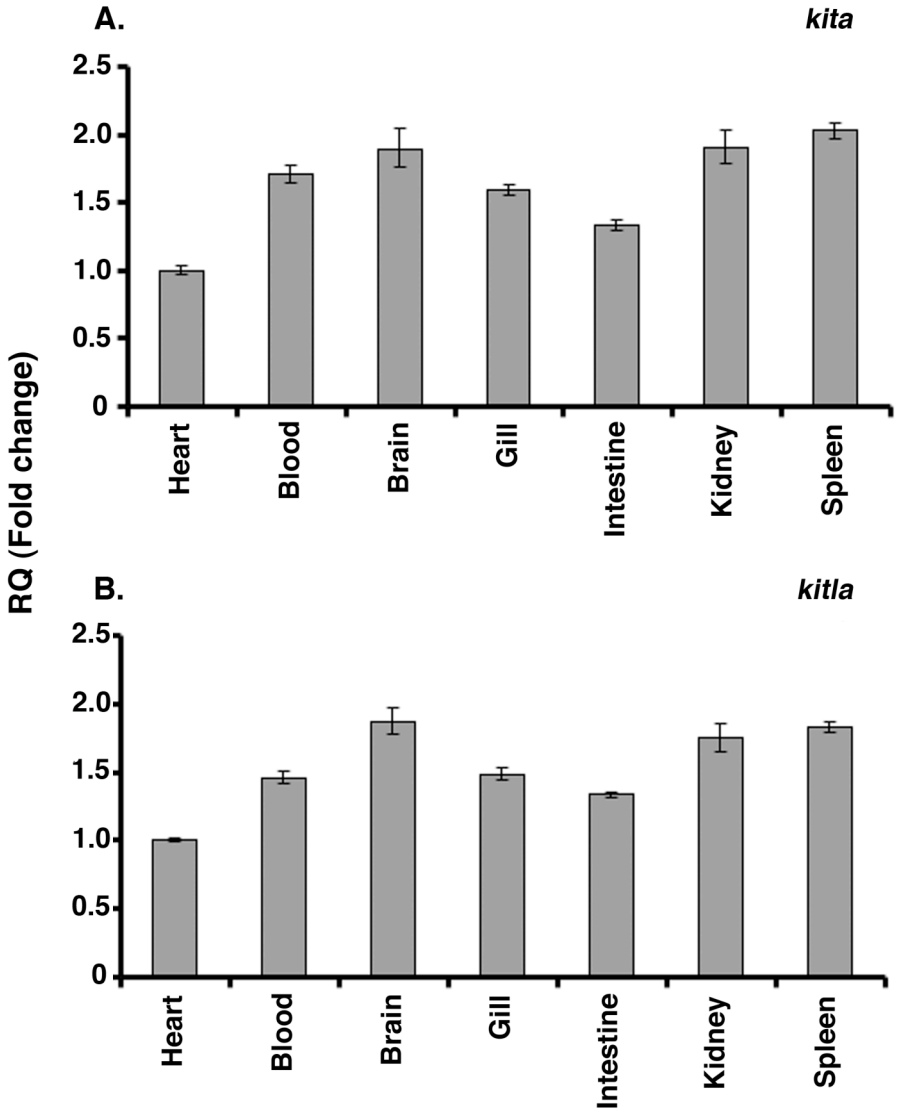


Figure 4.4: Examination of the expressions of goldfish *kita* and *kitla* in different goldfish tissues as determined by quantitative PCR.
Goldfish *kita* (A) and *kitla* (B) expressions were highest in body kidney, spleen, and brain with lower expression in the blood, gill, intestine and heart. Data was normalized to the heart and error bars are shown (n = 4).

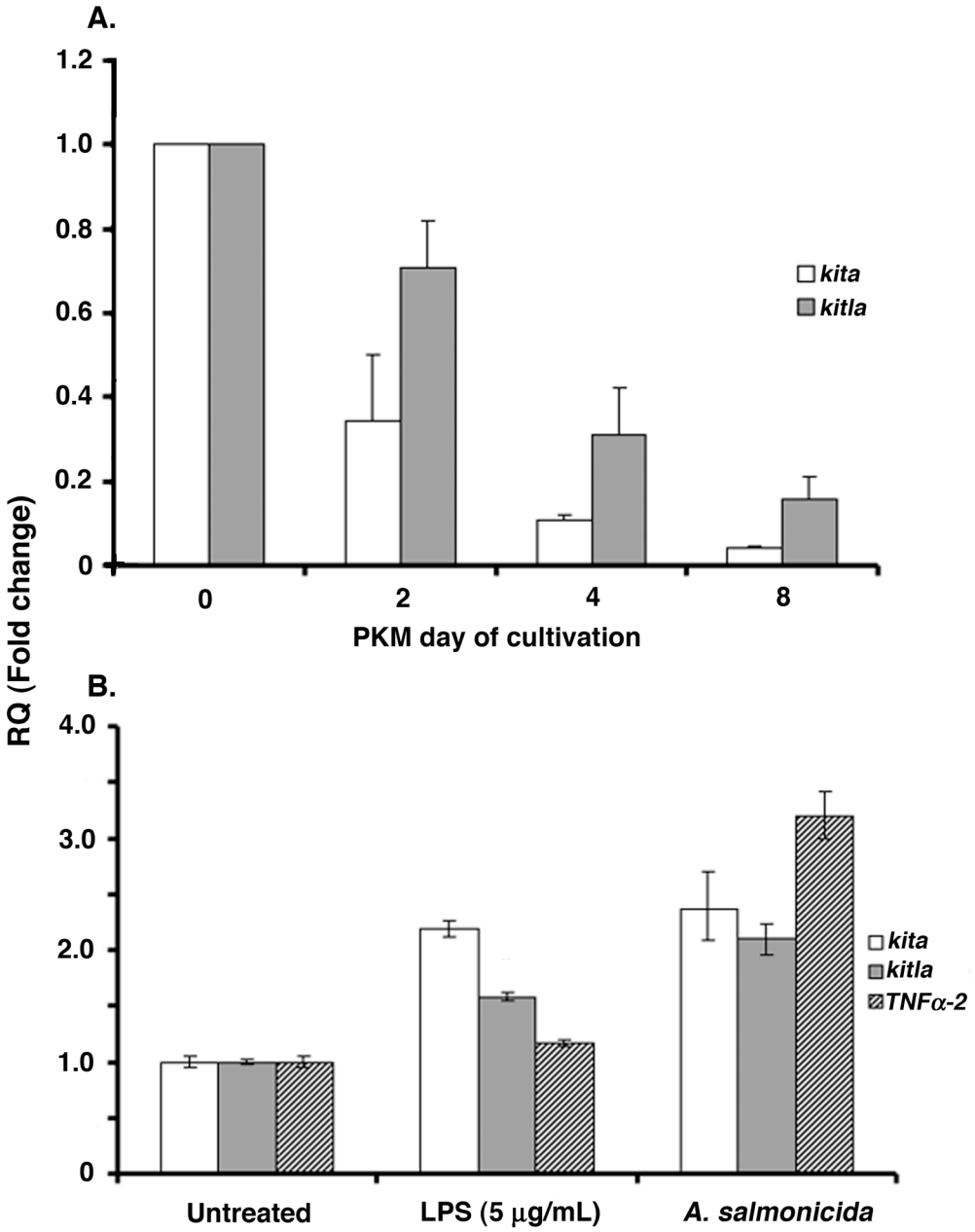


Figure 4.5: Comparison of the expressions of goldfish *kita* and *kitla* in PKM cultures over time and in activated day 8 PKMs as determined by quantitative PCR.

(A) Cells were seeded at 1 x10^6 cells per well in complete NMGFL-15 medium from individual fish. Data was normalized to day 0 cultures and error bars represent standard error (n = 3). (B) Day 8 PKM mononuclear cells from four fish were pooled and seeded at 1 x 10^7 cells/well. Cells were treated with 5 μg/mL of LPS (Sigma) or a 1:200 dilution of heat-killed *A. salmonicida* stock for 24 hours. Triplicate plates were run. Standard error between runs is shown.

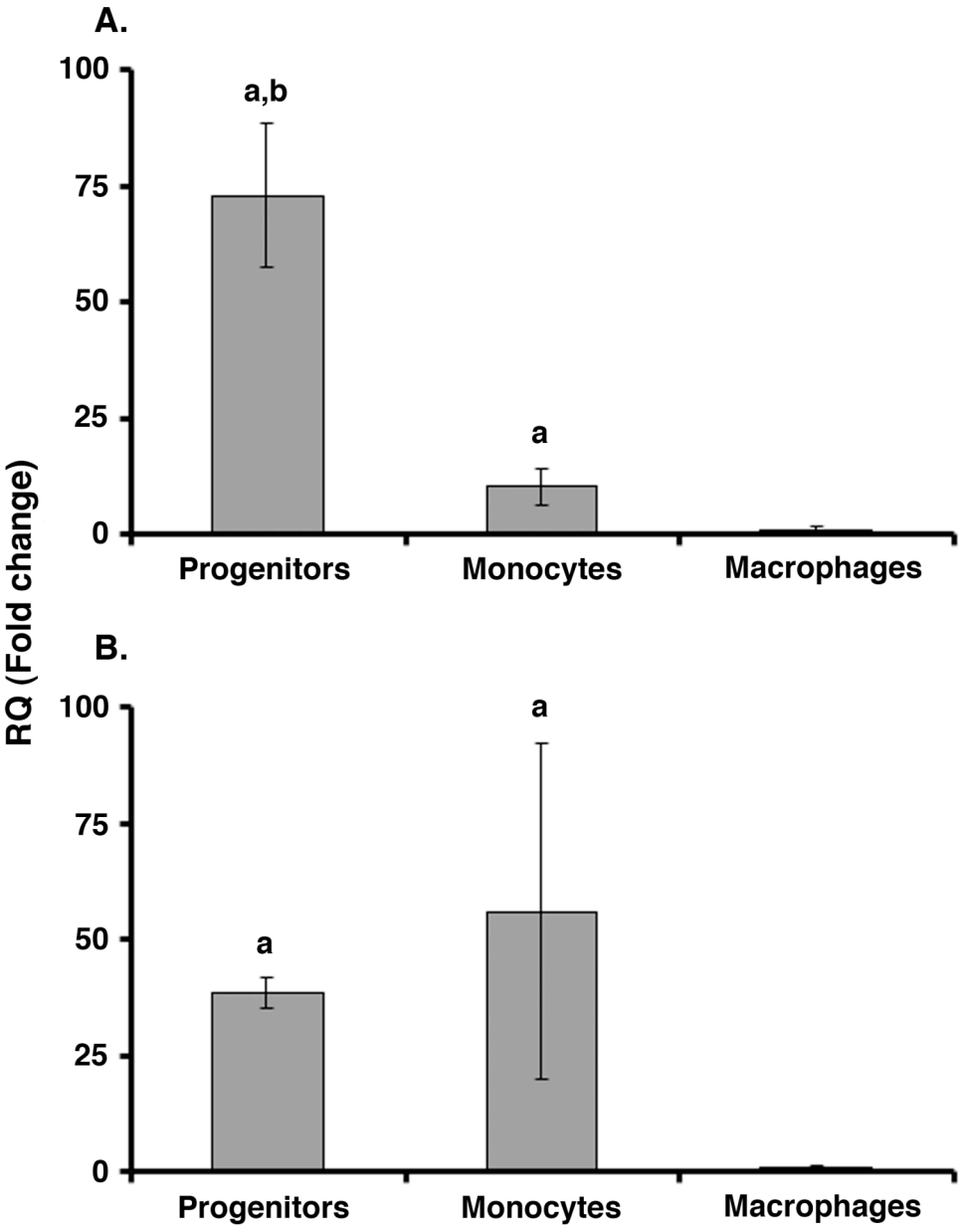


Figure 4.6: Examination of the expressions of goldfish *kita* and *kitla* in sorted progenitors, monocytes, and macrophages as determined by quantitative PCR.
(A) Expression of goldfish *kita* was highest in sorted progenitor cells with minimal expression in monocytes and macrophages. (B) Goldfish *kitla* was highly expressed in both sorted progenitors and monocytes. Data was normalized to the sorted macrophage reference sample, error bars are shown, and (a) denotes significance compared to the macrophages, (b) denotes significance compared to the monocyte sample (n = 3).

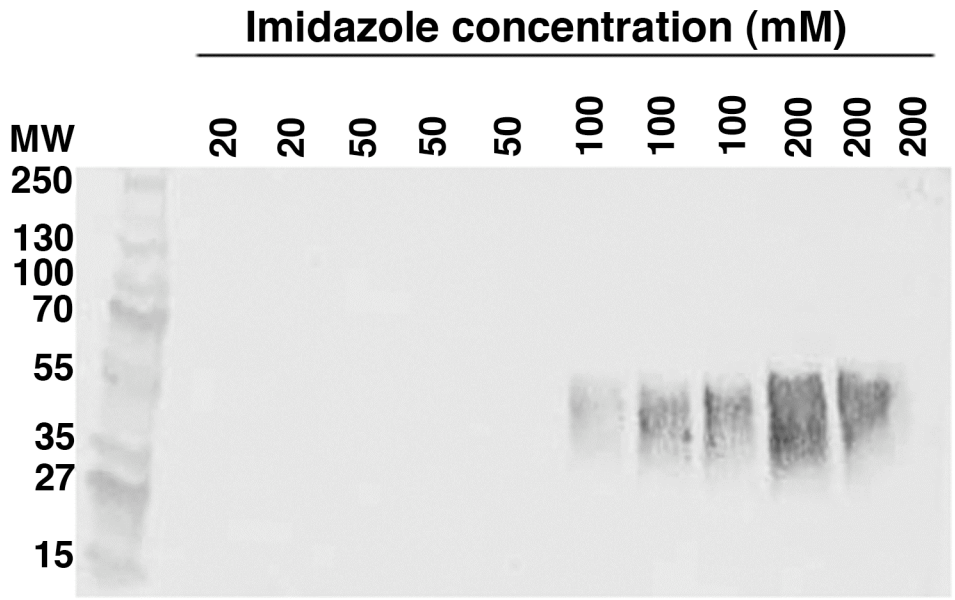


Figure 4.7: Western blot showing protein expression and purification of recombinant goldfish KITLA in HEK293T cell culture supernatants.
NiNTA beads were washed stringently with 20 mM imidazole wash buffer prior to elution in a step-wise gradient. Recombinant protein was eluted at a 100 mM imidazole concentration or higher. MW - molecular weight markers.

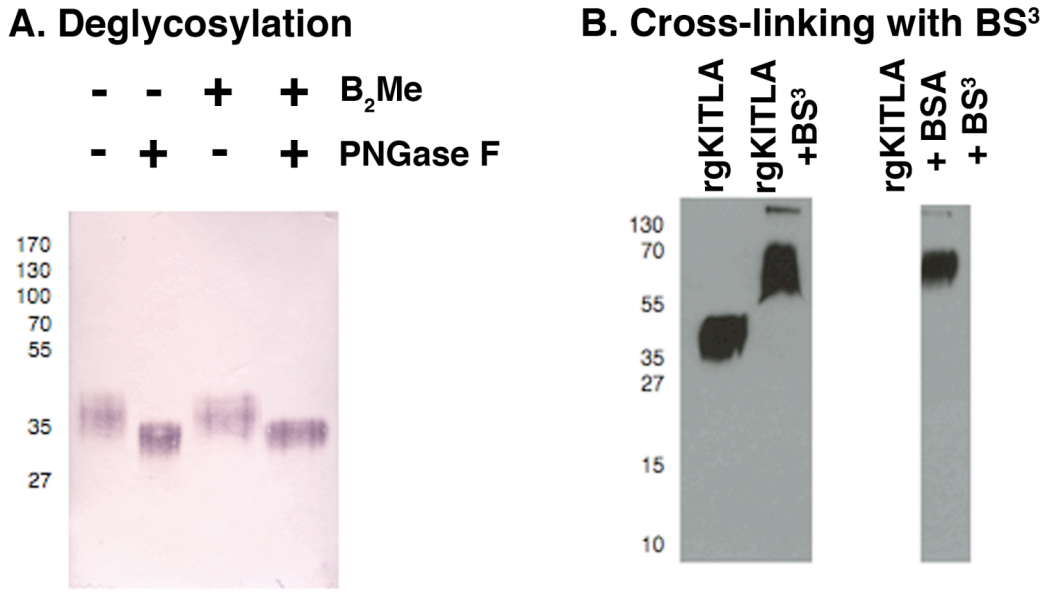


Figure 4.8: Recombinant goldfish KITLA is glycosylated and forms homodimers and tetramers in solution.
Recombinant goldfish KITLA was incubated with or without PNGase F to cleave N-linked glycosylations (A). The association of rgKITLA in solution was assessed by incubating rgKITLA in the absence of the cross-linking agent BS^3 (negative control), in the presence of BS^3, or in the presence of BS^3 and bovine serum albumin (BSA) to demonstrate specificity of the protein interactions (B).

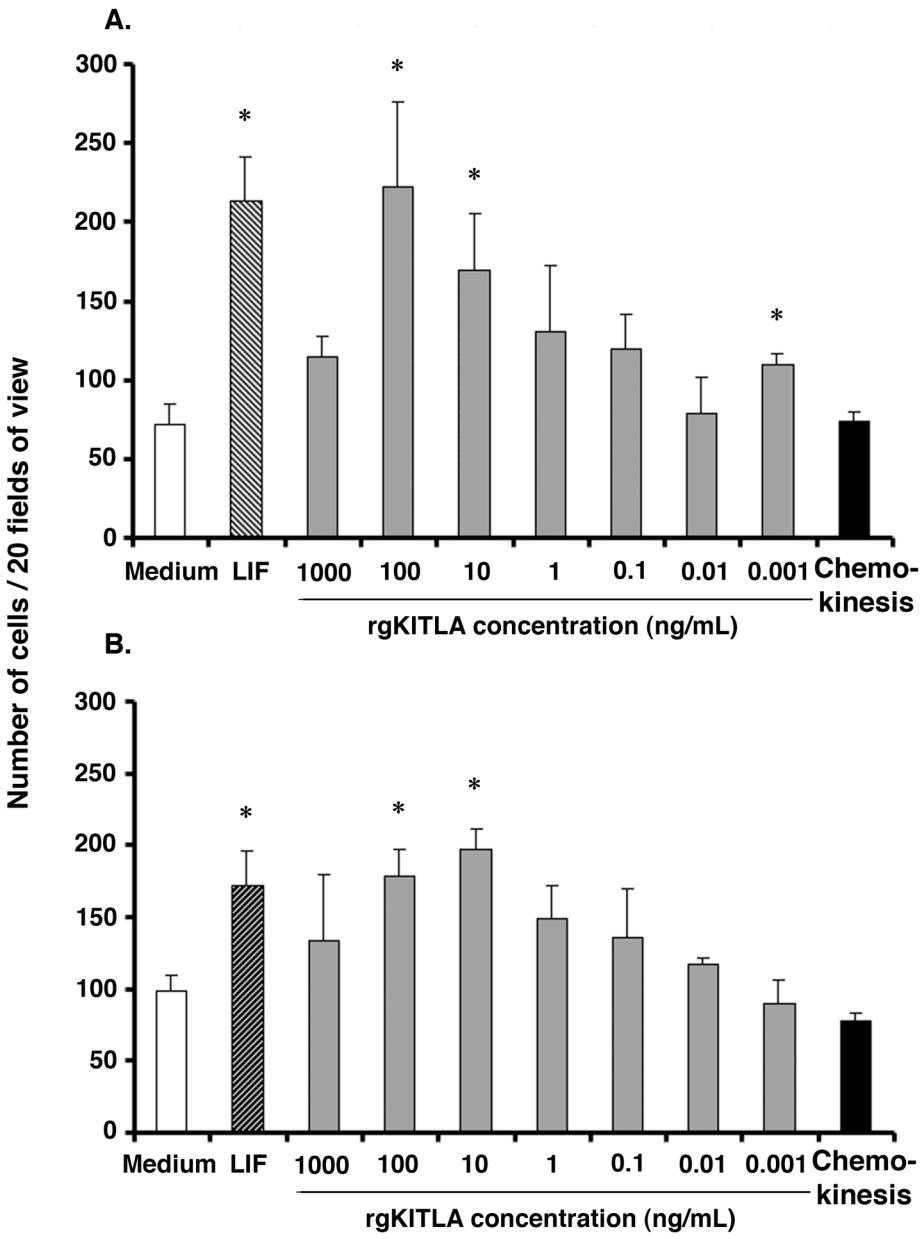


Figure 4.9: Recombinant goldfish KITLA induces chemotaxis in day 2 and day 6-9 PKM mononuclear cells.

Following a four-hour incubation, filters were stained with Gills Solution 3 and the total number of cells in 20 random fields of view under oil immersion (100X) were counted. The negative control was medium alone and the positive control was 10 ng/mL goldfish recombinant leukemia inhibitory factor (rgLIF). The chemoattractive nature of different concentrations of recombinant goldfish KITLA (rgKITLA) to (A) day 2 and (B) day 6-9 PKM mononuclear cells was assessed (n = 4). Asterisks denote statistical significance, $P < 0.05$. Note that rgKITLA produced using a prokaryotic expression system did not induce a chemotactic response, while rgKITLA produced using an eukaryotic expression system did, as shown above.

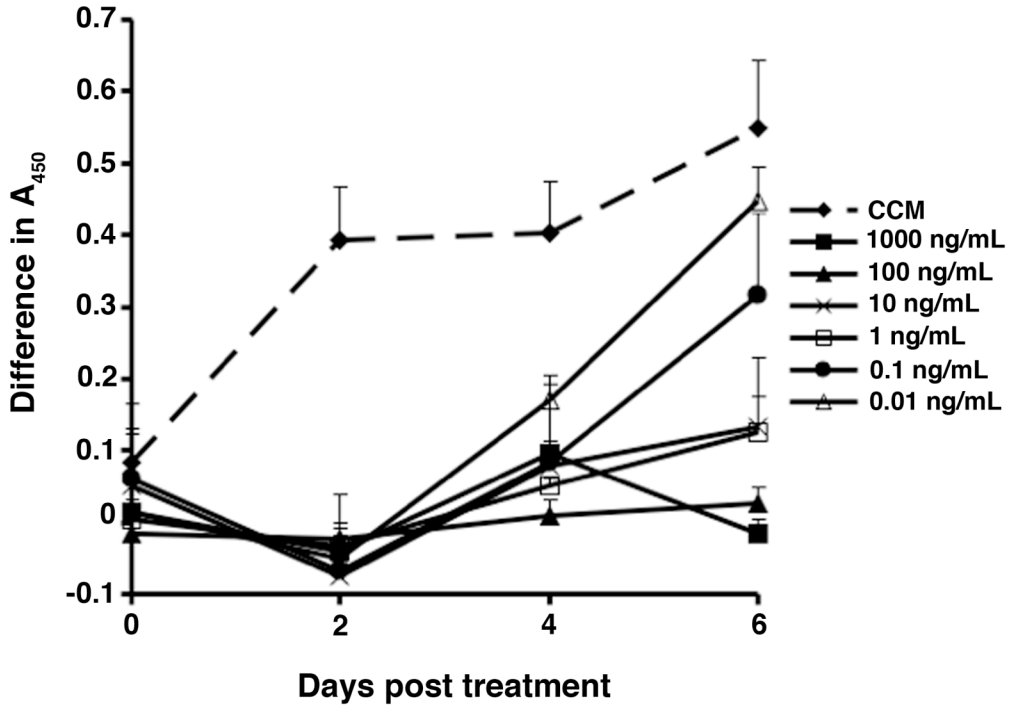


Figure 4.10: Recombinant goldfish KITLA induces proliferation of day 2 PKM cultures.

Recombinant goldfish KITLA concentrations of 0.01 ng/mL and 0.1 ng/mL induced a proliferative response in day 2 PKM leukocytes. Cells treated with incomplete NMGLF-15 medium were subtracted from experimental groups to control for the production of endogenous growth factors. CCM-cell conditioned medium was used as a positive control for proliferation and proliferation values were compared to cells treated with CCM. Error bars represent standard error (n = 4).

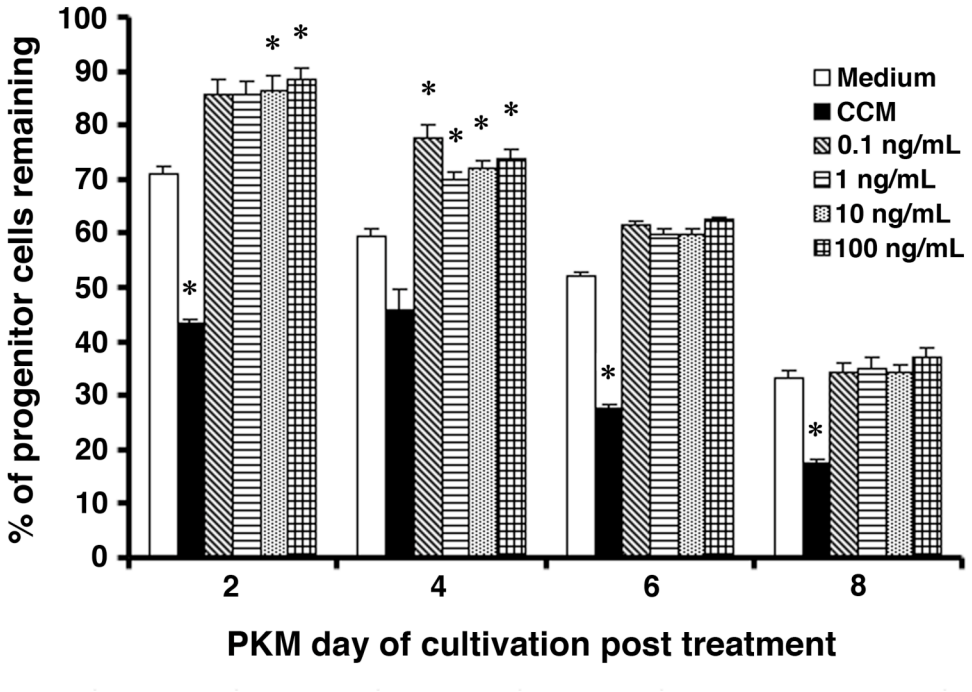


Figure 4.11: Recombinant goldfish KITLA promotes the survival of progenitor cells.
Day 2 PKM mononuclear cells were seeded at a concentration of 1 x 10^6 cells/mL in incomplete medium (medium), cell conditioned medium (CCM) or various concentrations of recombinant goldfish KITLA. Flow cytometry analysis was performed at 0, 2, 4, 6, and 8 days post treatment and the number of progenitor cells remaining compared to the initial number of progenitor cells expressed as a percentage of progenitor cells remaining. Error bars are representative of standard error, and (*) denotes significance ($P < 0.05$) when compared to a time matched medium control (n = 3).

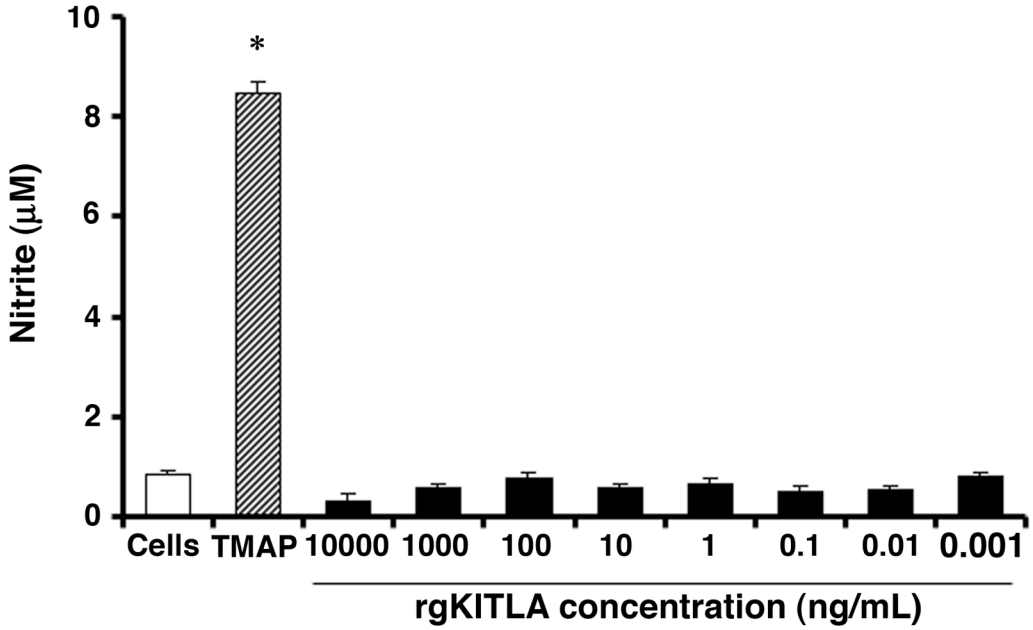


Figure 4.12: Recombinant goldfish KITLA does not induce a nitric oxide response in mature macrophages.
Day 6-8 primary kidney macrophages were incubated with medium alone (cells, negative control), transferrin macrophage activating peptide (TMAP, positive control), or varying concentrations of rgKITLA for 48 hours. Following incubation, supernatants were assessed for the production of nitrite using the Griess reaction (n = 4). Error bars are representative of standard error, and (*) denotes significance ($P < 0.05$).

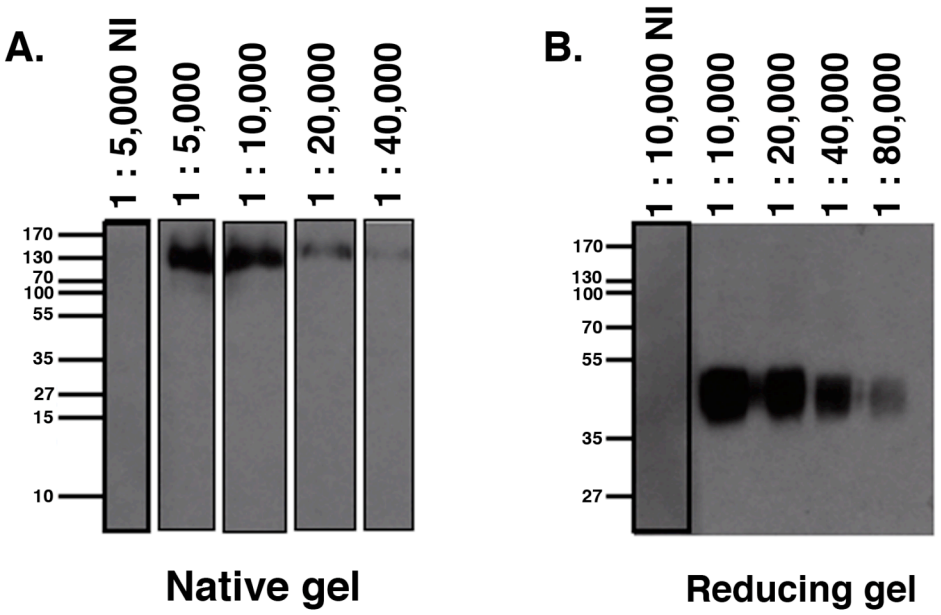


Figure 4.13: Anti-recombinant goldfish KITLA antibody recognizes rgKITLA under native and denaturing and reducing conditions.
Recombinant gKILTA was run under native (A) or denaturing and reducing (B) conditions. Individual nitrocellulose strips were blotted with serial dilutions of the affinity purified rabbit anti-rgKITLA antibody. The stock solution of the anti-rgKITLA antibody was at a stock concentration of 256 μg/mL. A dilution of non-immune (NI) rabbit IgG, at the same stock concentration, was used and diluted to the lowest dilution tested for the anti-rgKITLA antibody. The NI blot shows no antibody recognition of the rgKITLA protein.

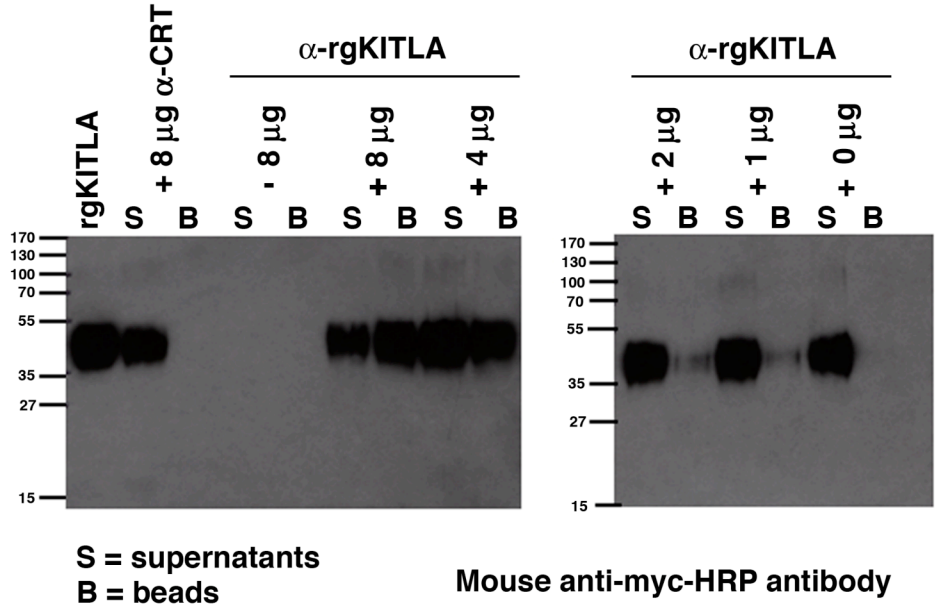


Figure 4.14: Immunoprecipitation of rgKITLA by the anti-rgKITLA antibody. Recombinant goldfish KITLA (2 *µ*g) was incubated with 8 *µ*g of anti-CRT (negative control) or with varying concentrations of anti-rgKITLA antibody. Following overnight incubation, sepharose G beads were added and incubated for an additional hour. Supernatants (S) were collected, and beads washed before they were boiled in SDS-PAGE loading buffer (B). Supernatant (S) and bead (B) fractions were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were probed with an anti-myc-HRP antibody to detect the presence of rgKITLA.

4.4 References

[1] **Ashman LK.** The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol. 1999;31(10):1037-51.

[2] **Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C.** Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. Clin Cancer Res. 2008;14(7):1926-30.

[3] **Roskoski R, Jr.** Signaling by Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun. 2005;337(1):1-13.

[4] **Mauduit C, Hamamah S, Benahmed M.** Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update. 1999;5(5):535-45.

[5] **Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, et al.** The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. Dev Suppl. 1993125-37.

[6] **Reid K, Nishikawa S, Bartlett PF, Murphy M.** Steel factor directs melanocyte development in vitro through selective regulation of the number of c-kit^{+} progenitors. Dev Biol. 1995;169(2):568-79.

[7] **Wehrle-Haller B, Weston JA.** Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. Development. 1995;121(3):731-42.

[8] **Yee NS, Paek I, Besmer P.** Role of kit-ligand in proliferation and suppression of apoptosis in mast cells: basis for radiosensitivity of white spotting and steel mutant mice. J Exp Med. 1994;179(6):1777-87.

[9] **Serve H, Yee NS, Stella G, Sepp-Lorenzino L, Tan JC, Besmer P.** Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. EMBO J. 1995;14(3):473-83.

[10] **Yee NS, Langen H, Besmer P.** Mechanism of kit ligand, phorbol ester, and calcium-induced down-regulation of c-kit receptors in mast cells. J Biol Chem. 1993;268(19):14189-201.

[11] **Wershil BK, Tsai M, Geissler EN, Zsebo KM, Galli SJ.** The rat c-kit ligand, stem cell factor, induces c-kit receptor-dependent mouse mast cell activation in vivo. Evidence that signaling through the c-kit receptor can induce expression of cellular function. J Exp Med. 1992;175(1):245-55.

[12] **Russell ES.** Hereditary anemias of the mouse: a review for geneticists. Adv Genet. 1979;20357-459.

[13] **Besmer P, Murphy JE, George PC, Qiu FH, Bergold PJ, Lederman L, et al.** A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature. 1986;320(6061):415-21.

[14] **Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, et al.** Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. 1987;6(11):3341-51.

[15] **Fantl WJ, Johnson DE, Williams LT.** Signalling by receptor tyrosine kinases. Annu Rev Biochem. 1993;62453-81.

[16] **Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, et al.** The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell. 1990;63(1):225-33.

[17] **Copeland NG, Gilbert DJ, Cho BC, Donovan PJ, Jenkins NA, Cosman D, et al.** Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell. 1990;63(1):175-83.

[18] **Witte ON.** Steel locus defines new multipotent growth factor. Cell. 1990;63(1):5-6.

[19] **Zhang Z, Zhang R, Joachimiak A, Schlessinger J, Kong XP.** Crystal structure of human stem cell factor: implication for stem cell factor receptor dimerization and activation. Proc Natl Acad Sci U S A. 2000;97(14):7732-7.

[20] **Arakawa T, Yphantis DA, Lary JW, Narhi LO, Lu HS, Prestrelski SJ, et al.** Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. J Biol Chem. 1991;266(28):18942-8.

[21] **Jiang X, Gurel O, Mendiaz EA, Stearns GW, Clogston CL, Lu HS, et al.** Structure of the active core of human stem cell factor and analysis of binding to its receptor kit. EMBO J. 2000;19(13):3192-203.

[22] **Philo JS, Wen J, Wypych J, Schwartz MG, Mendiaz EA, Langley KE.** Human stem cell factor dimer forms a complex with two molecules of the extracellular domain of its receptor, Kit. J Biol Chem. 1996;271(12):6895-902.

[23] **Broudy VC.** Stem cell factor and hematopoiesis. Blood. 1997;90(4):1345-64.

[24] **Blechman JM, Lev S, Brizzi MF, Leitner O, Pegoraro L, Givol D, et al.** Soluble c-kit proteins and antireceptor monoclonal antibodies confine the binding site of the stem cell factor. J Biol Chem. 1993;268(6):4399-406.

[25] **Lev S, Blechman J, Nishikawa S, Givol D, Yarden Y.** Interspecies molecular chimeras of kit help define the binding site of the stem cell factor. Mol Cell Biol. 1993;13(4):2224-34.

[26] **Yuzawa S, Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J.** Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell. 2007;130(2):323-34.

[27] **Galli SJ, Zsebo KM, Geissler EN.** The kit ligand, stem cell factor. Adv Immunol. 1994;551-96.

[28] **Parichy DM, Rawls JF, Pratt SJ, Whitfield TT, Johnson SL.** Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for

hematopoiesis or primordial germ cell development. Development. 1999;126(15):3425-36.

[29] **Mellgren EM, Johnson SL.** kitb, a second zebrafish ortholog of mouse Kit. Dev Genes Evol. 2005;215(9):470-77.

[30] **Hultman KA, Bahary N, Zon LI, Johnson SL.** Gene Duplication of the zebrafish kit ligand and partitioning of melanocyte development functions to kit ligand a. PLoS Genet. 2007;3(1):e17.

[31] **Rawls JF, Johnson SL.** Zebrafish kit mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration. Development. 2000;127(17):3715-24.

[32] **Rawls JF, Johnson SL.** Requirements for the kit receptor tyrosine kinase during regeneration of zebrafish fin melanocytes. Development. 2001;128(11):1943-9.

[33] **Rawls JF, Johnson SL.** Temporal and molecular separation of the kit receptor tyrosine kinase's roles in zebrafish melanocyte migration and survival. Dev Biol. 2003;262(1):152-61.

[34] **Mellgren EM, Johnson SL.** A requirement for kit in embryonic zebrafish melanocyte differentiation is revealed by melanoblast delay. Dev Genes Evol. 2004;214(10):493-502.

[35] **Zandstra PW, Lauffenburger DA, Eaves CJ.** A ligand-receptor signaling threshold model of stem cell differentiation control: a biologically conserved mechanism applicable to hematopoiesis. Blood. 2000;96(4):1215-22.

[36] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[37] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[38] **Belosevic M, Hanington PC, Barreda DR.** Development of goldfish macrophages in vitro. Fish Shellfish Immunol. 2006;20(2):152-71.

[39] **Summerfield A, Horn MP, Lozano G, Carrasco CP, Atze K, McCullough K.** C-kit positive porcine bone marrow progenitor cells identified and enriched using recombinant stem cell factor. J Immunol Methods. 2003;280(1-2):113-23.

[40] **Rosnet O, Birnbaum D.** Hematopoietic receptors of class III receptor-type tyrosine kinases. Crit Rev Oncog. 1993;4(6):595-613.

[41] **Keshet E, Lyman SD, Williams DE, Anderson DM, Jenkins NA, Copeland NG, et al.** Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. EMBO J. 1991;10(9):2425-35.

[42] **Torihashi S, Ward SM, Nishikawa S, Nishi K, Kobayashi S, Sanders KM.** c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res. 1995;280(1):97-111.

[43] **Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A.** W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. Nature. 1995;373(6512):347-9.

[44] **Natali PG, Nicotra MR, Sures I, Santoro E, Bigotti A, Ullrich A.** Expression of c-kit receptor in normal and transformed human nonlymphoid tissues. Cancer Res. 1992;52(22):6139-43.

[45] **Lammie A, Drobnjak M, Gerald W, Saad A, Cote R, Cordon-Cardo C.** Expression of c-kit and kit ligand proteins in normal human tissues. J Histochem Cytochem. 1994;42(11):1417-25.

[46] **Kent DG, Dykstra BJ, Cheyne J, Ma E, Eaves CJ.** Steel factor coordinately regulates the molecular signature and biologic function of hematopoietic stem cells. Blood. 2008;112(3):560-7.

[47] **Roskoski R, Jr.** Structure and regulation of Kit protein-tyrosine kinase--the stem cell factor receptor. Biochem Biophys Res Commun. 2005;338(3):1307-15.

[48] **Barreda DR, Hanington PC, Belosevic M.** Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol. 2004;28(5):509-54.

[49] **Barreda DR, Neumann NF, Belosevic M.** Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Dev Comp Immunol. 2000;24(4):395-406.

[50] **Kobayashi I, Sekiya M, Moritomo T, Ototake M, Nakanishi T.** Demonstration of hematopoietic stem cells in ginbuna carp (*Carassius auratus langsdorfii*) kidney. Dev Comp Immunol. 2006;30(11):1034-46.

[51] **Kobayashi I, Kuniyoshi S, Saito K, Moritomo T, Takahashi T, Nakanishi T.** Long-term hematopoietic reconstitution by transplantation of kidney hematopoietic stem cells in lethally irradiated clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Dev Comp Immunol. 2008;32(8):957-65.

[52] **Faust EA, Saffran DC, Toksoz D, Williams DA, Witte ON.** Distinctive growth requirements and gene expression patterns distinguish progenitor B cells from pre-B cells. J Exp Med. 1993;177(4):915-23.

[53] **Hanington PC, Wang T, Secombes CJ, Belosevic M.** Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[54] **Hanington PC, Barreda DR, Belosevic M.** A novel hematopoietic granulin induces proliferation of goldfish (*Carassius auratus* L.) macrophages. J Biol Chem. 2006;281(15):9963-70.

[55] **Hanington PC, Belosevic M.** Characterization of the leukemia inhibitory factor receptor in the goldfish (*Carassius auratus*). Fish Shellfish Immunol. 2005;18(5):359-69.

[56] **Winkler IG, Levesque JP.** Mechanisms of hematopoietic stem cell mobilization: when innate immunity assails the cells that make blood and bone. Exp Hematol. 2006;34(8):996-1009.

[57] **Young SM, Cambareri AC, Odell A, Geary SM, Ashman LK.** Early myeloid cells expressing c-KIT isoforms differ in signal transduction, survival and chemotactic responses to Stem Cell Factor. Cell Signal. 2007;19(12):2572-81.

[58] **Pietsch T, Kyas U, Steffens U, Yakisan E, Hadam MR, Ludwig WD, et al.** Effects of human stem cell factor (c-kit ligand) on proliferation of myeloid leukemia cells: heterogeneity in response and synergy with other hematopoietic growth factors. Blood. 1992;80(5):1199-206.

[59] **Kuriu A, Ikeda H, Kanakura Y, Griffin JD, Druker B, Yagura H, et al.** Proliferation of human myeloid leukemia cell line associated with the tyrosine-phosphorylation and activation of the proto-oncogene c-kit product. Blood. 1991;78(11):2834-40.

[60] **Hanington PC, Belosevic M.** Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. Dev Comp Immunol. 2007;31(8):817-29.

[61] **Ogawa M, Nishikawa S, Yoshinaga K, Hayashi S, Kunisada T, Nakao J, et al.** Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo. Development. 1993;117(3):1089-98.

Chapter 5: Characterization of myeloid progenitor cell surface markers of the goldfish[2]

5.1 Introduction

The process of myeloid cell differentiation from a HSC to a mature cell type is an exquisitely fine-tuned process that requires the coordination at transcriptional and translational levels. Coordination of gene expression includes those that encode for surface proteins required for interaction with the hematopoietic cell niche or surface receptors that bind hematopoietic growth factors. The presence or absence of certain surface proteins on progenitor cells has been used in mammalian systems to isolate progenitor cells at particular stages of cell differentiation through the use of antibodies directed against surface proteins. However, antibodies to such surface proteins in teleost systems are not available and lack of antibodies to fish proteins impede the acquisition of relatively homogenous subpopulations of progenitor cells at distinct cell junctures of differentiation. Therefore, I focused on identifying and characterizing four surface proteins as candidate markers of goldfish early progenitor cells; prominin and KIT (identification and characterization of KIT was discussed in chapter 4) and markers of committed myeloid cells, CSF-1R and GCSFR (the identification and molecular characterization of GCSFR is the subject of chapter 8). The production and characterization of anti-prominin and anti-CSF-1R antibodies are the focus of this chapter.

[2] A version of this chapter has been published: Katzenback and Belosevic, 2012. *Fish and Shellfish Immunology* 32:434-435

Studies by Yin et al. [1] identified a monoclonal antibody, AC133, that bound to an antigen expressed on $CD34^+$ cells, both non committed $CD34^+$ cells, and $CD34^+$ cells that had committed to the granulomonocytic pathway. The ability of AC133 to recognize these cell populations decreased as the cells underwent granulomonocytic differentiation [1]. The human antigen recognized by AC133 was later identified as prominin-1 [2]. Prominin-1 is a novel 5-transmembrane protein with two large extracellular loops possessing 8 potential glycosylation sites, an extracellular N-terminus, and an intracellular C-terminus [2]. While immunohistochemical studies suggest that the AC133 antibody recognized $CD34^+$ bright cells, northern blotting resulted in the identification of prominin mRNA in pancreas, kidney and placental tissues [2], suggesting that prominin-1 is a marker for all tissue stem cells. In all of these tissues, prominin was associated with membrane microdomains, such as the plasma membrane protrusions in stem cells [3], the microvilli of epithelial cells [3, 4], and the plasma membrane evaginations of rod photoreceptor cells [5]. The function of prominin-1 is not known, however, a frame-shift mutation in prominin-1 caused retinal degeneration in mice [5].

A second member of the prominin family was identified, prominin-2, that co-localized with prominin-1 in tissues, and in plasma membrane microdomains [6]. Prominin-2 was similar in structure to prominin-1 and the alignment of both prominins revealed a consensus sequence known as the prominin signature: CXPX(12,13)CX(5)(P/S)X(4)WX(2)hhXh [6]. Where X is any residue and number of residues, and h is any hydrophobic residue. Both prominins are cholesterol-binding proteins in the plasma membrane [7, 8]. However, the ligands of prominins are not known.

Recently, a prominin-like transcript was identified in goldfish [9, 10]. The goldfish prominin showed equal identity, ~27%, to mouse prominin-1 and prominin-2, and quantitative PCR demonstrated that sorted goldfish progenitor cells had higher mRNA levels of *prominin*, compared to those of sorted monocytes and macrophages [10]. Based on these initial studies, prominin appeared to be a potential marker of early progenitor cells.

Macrophages play critical roles in maintaining homeostasis and as central cells of the innate immune response under normal and “emergency” conditions. Therefore, the continual production of macrophages from hematopoietic precursors is required to maintain a variety of physiological processes within an organism. The central growth factor and receptor that regulate the survival, proliferation, and development of macrophages and their precursors is colony-stimulating factor-1 (CSF-1) and its’ type III tyrosine kinase receptor, CSF-1R [11-14]. The binding of homodimeric CSF-1 to membrane CSF-1R (mCSF-1R) results in homodimerization of mCSF-1R leading to autophosphorylation of the intracellular tyrosine residues and activation of JAK/STAT, PI3K/Akt, and MAP kinase pathways, among others [15, 16]. Until recently, mononuclear phagocyte development appeared to be mediated by CSF-1. However, another ligand of CSF-1R, IL-34, has been shown to contribute to macrophage development in mammals in addition to CSF-1 [17-19]. The importance of CSF-1R signaling during macrophages development has permitted CSF-1R to be used as a marker of the macrophage lineage (progenitors, monocytes and macrophages) in mammalian systems [13], as expression of CSF-1R progressively increases with macrophage development [20].

Since macrophages are found across all metazoan, it is not surprising that the molecules that regulate macrophage development are also conserved. CSF-1R sequences have been identified in a number of teleost species including puffer fish [21, 22], zebrafish [23], rainbow trout [24], gilthead seabream [25] and goldfish [26], and appears to be a marker of monocytes and macrophages in teleosts [25-27]. The Belosevic lab has previously developed a unique *in vitro* derived primary kidney macrophage (PKM) culture system in which all three populations (progenitor cells, monocytes, and mature macrophages) are present [28] in which we can study macrophage development. Based on previous studies in the laboratory, goldfish PKMs appear to independently regulate their own development in a manner unique from a mammalian system: (1) they are capable of producing their own endogenous growth factors and progenitor cells can develop into fully functional monocytes and macrophages in vitro [28-30] and (2) senescence phase macrophages produce a soluble form of the CSF-1R, sCSF-1R, through alternative splicing that is believed to be involved in the regulation of CSF-1 signaling through mCSF-1R [26, 31]. While recombinant goldfish CSF-1 has been shown to induce monocyte proliferation and differentiation [31, 32] and aid in the long-term survival of mature macrophages in culture [32], limited studies have been performed on teleost progenitor cells in terms of CSF-1/CSF-1R function. To date, there are no reports on the expression of mCSF-1R on teleost progenitor cells committed to the macrophage lineage.

In this chapter, I describe the work performed to produce and validate antibodies to goldfish prominin and CSF-1R for potential use in identifying subpopulations of goldfish myeloid progenitor cells. The objectives of this study were to (1) produce a recombinant prominin protein, (2) produce a polyclonal

antibody to the recombinant prominin protein, (3) characterize the binding of the anti-prominin antibody to recombinant and native molecules, (4) characterize an antibody to goldfish sCSF-1R, (5) examine the distribution of CSF-1R$^+$ cells from goldfish tissues, and lastly (6) determine whether CSF-1R is expressed on the surface of goldfish progenitor cells and how this population of CSF-1R$^+$ progenitor cells changes over time of culture.

5.2 Results

5.2.1 Quantitative PCR expression of *prominin* in goldfish tissues

Goldfish PROMININ was predicted to have a pentaspan transmembrane structure with an extracellular amino terminus, two large extracellular loops, and an intracellular carboxy terminus (Fig. 5.1). The extracellular loops are predicted to have ten predicted N-linked glycosylation sites and a prominin signature sequence has been identified in goldfish prominin, similar to the other prominin family members [10]. To confirm the constitutive expression of *prominin* in tissues, the kidney, spleen, gill, heart, brain and intestine tissues were harvested from four animals and used to assess mRNA levels using quantitative PCR. Goldfish *prominin* mRNA levels were similar in all tissues examined (Fig. 5.2) and *prominin* mRNA levels were shown to be highest in sorted progenitor cells [10], suggesting that goldfish prominin may be a marker of early progenitor cells in goldfish.

5.2.2 Expression and purification of recombinant PROMININ extracellular loop-1

The nucleotide sequence corresponding to the first large extracellular loop of PROMININ (Fig. 5.1) was cloned into the pET151 expression vector. The construct was sequenced to ensure proper insertion and reading frame. The construct encoded for a 6x his tag to allow for protein purification, a V5 epitope, the first extracellular loop of prominin and a stop codon. The construct was transformed into BL21star *E. coli*. Bacteria were grown up overnight in 10 mL of LB containing 100 μg/mL of ampicillin and a 1:100 dilution of the overnight culture used for the pilot expression study. Bacteria were grown for 1 hour prior to induction with 0.1 mM or 1 mM of IPTG, and samples of bacteria taken at 1hr, 2 hrs, 3 hrs, and 4 hrs post induction (Fig. 5.3A). Samples were centrifuged to pellet the bacteria, and the cells lysed in the presence of protease inhibitors. Samples were run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted with a 1:5000 dilution of anti-his antibody followed by incubation with a 1:3000 dilution of a goat anti-mouse-AP antibody (Fig. 5.3A). Recombinant goldfish PROMININ extracellular loop-1 (rgPECL-1) was detected at both concentrations of IPTG induction at all time points post induction (Fig. 5.3A). An induction time of 4 hours at a 1 mM IPTG induction concentration was chosen for large-scale protein production. Following large-scale production, bacteria were lysed under denaturing conditions and the recombinant protein purified using MagneHis beads. The purified recombinant protein was run on an SDS-PAGE gel and silver stained to detect total protein (Fig. 5.3B, left panel). A duplicate gel was transferred to nitrocellulose membrane and blotted with an anti-his antibody, followed by a secondary goat anti-mouse-AP antibody (Fig. 5.3B,

right panel). Silver staining revealed three bands; a doublet at an approximate molecular weight of 31-35 kDa, and a third band at ~20 kDa (Fig. 5.3B, left panel). All three of these bands were recognized by the anti-His antibody (Fig. 5.3B, right panel). Mass spectrometry confirmed that all three bands were PECL-1. These bands likely represent truncated proteins that are known to occur in prokaryotic expression systems.

5.2.3 Production of a polyclonal antibody to rgPECL-1

Purified rgPECL-1 was used in the production of polyclonal antibodies from two rabbits. Rabbits were immunized using 150 μg of rgPECL-1 in Freunds complete adjuvant, followed by three subsequent booster injections containing 100 μg of rgPECL-1 in Freunds incomplete adjuvant. Rabbits were bled prior to immunization (non-immune serum) and after all booster injections and the test serum titre determined. After three booster injections, rabbits were bleed and the serum was collected (immune serum). The ability of anti-rgPECL-1 to recognize rgPECL-1 was determined using bacterial lysates expressing the rgPECL-1 protein. Immune serum at a 1:5000 dilution recognized rgPECL-1 in a western blot (Fig. 5.4A). The non-immune serum appeared to cross-react with a protein of ~35 kDa in the rgPECL-1/*E. coli* cell lysates (Fig. 5.4B).

In an attempt to detect the native PROMININ protein, day 2 PKMs were lysed, run in multiple lanes of an SDS-PAGE gel, transferred to a nitrocellulose membrane, strips incubated with a 1:1000 dilution of non-immune rabbit serum or anti-rgPECL-1 serum at a 1:1000 dilution, a 1:2500 dilution, or a 1:5000 dilution. The secondary antibody consisted of a 1:3000 dilution of goat anti-rabbit-AP

antibody. No bands were observed on the nitrocellulose strip incubated with the non-immune serum, or the 1:2500 or 1:5000 dilution of anti-rgPECL-1 serum (data not shown). However, a band at 90-97 kDa was observed on the nitrocellulose strip incubated with a 1:1000 dilution of anti-rgPECL-1 serum (Fig. 5.4C). The observed band was similar in molecular weight to the predicted weight of the full-length goldfish PROMININ protein without glycosylation. The identified band likely represents a cytoplasmic protein that has not undergone glycosylation.

Attempts were made to use the anti-rgPECL-1 serum to recognize membrane proteins from day 2 PKMs, however, no proteins were recognized following western blotting. In addition, the anti-PECL-1 antibody did not appear to recognize surface proteins when immunofluorescence studies were performed on PKM cells (data not shown). I believe the lack of anti-rgPECL-1 recognition of a native PROMININ molecule may be due to the extensive glycosylation predicted to occur for this protein. Optimization of deglycosylation procedures on live cells or isolated membrane proteins will be required in order to use this antibody to detect the native PROMININ molecule.

Lastly, to determine whether the anti-rgPECL-1 antibody could be used in immunoprecipitation studies, the ability of anti-rgPECL-1 to pull down rgPECL-1 from solution was assessed. Four hundred nanograms of rgPECL-1 were incubated with anti-recombinant *Trypanosoma carassii* calreticulin (anti-CRT, negative control) or varying concentrations (0 μg, 1 μg, 2 μg, 4 μg and 8 μg) of anti-rgPECL-1 for 2 hours. To the protein-antibody mixture, Protein G beads were added and incubated overnight. Following incubation, the supernatants were removed from the beads (S) and the beads were washed to remove unbound

protein, followed by the addition of SDS-PAGE loading buffer and boiling to dissociate antibody-protein complexes from the beads (B). The supernatant was run on a reducing gel along with a well containing 400 ng of rgPECL-1 as a loading control. Following transfer to a nitrocellulose membrane, blots were probed with an anti-V5 HRP antibody that recognizes the V5 epitope on the N-terminus of the rgPECL-1 protein (Fig. 5.5). The anti-CRT antibody did not bind rgPECL-1 as rgPECL-1 was only detected in the supernatant and not the bead fraction (Fig. 5.5, top panel). However, when beads were pre-incubated with 8 μg of anti-rgPECL-1, a large fraction of the total rgPECL-1 was detected in the bead fraction, suggesting that anti-rgPECL-1 bound rgPECL-1 (Fig. 5.5, top panel). The binding of rgPECL-1 by anti-rgPECL-1 decreased in a dose-dependent manner with decreasing concentrations of anti-rgPECL-1 (Fig. 5.5). Accordingly, there was an increase in the amount of rgPECL-1 detected in the supernatants with decreasing amounts of anti-rgPECL-1 antibody (Fig. 5.5). Non-specific binding of rgPECL-1 to the beads was not detected, as shown by the absence of rgPECL-1 in the bead fraction of the sample incubated with rgPECL-1 in the absence of anti-rgPECL-1 antibody (Fig. 5.5, lower panel).

5.2.4 Validation of the anti-sCSF-1R antibody

The secretion signal and the two extracellular immunoglobulin domains of the goldfish soluble CSF-1R (sCSF-1R), which are common to both the soluble and membrane bound forms of CSF-1R [26], were fused to the transmembrane sequence of the catfish FcRγL (IpFcRγL) chain by overlap extension PCR. The resulting sCSF-1R/IpFcRγL fusion was then cloned, and transfected into RBL

cells. This construct would produce a sCSF-1R/IpFcRγL fusion protein, lacking a hemagglutinin (HA) tag, on the surface of the RBL cells. As a positive control, RBL cells expressing the IpLITR/IpFcRγL fusion protein tagged with the N-terminal HA tag was used. One million cells were incubated with 20 μg/mL mouse IgG3, 20 μg anti-HA, 25 μg/mL rabbit IgG, or 25 μg/mL anti-sCSF-1R IgG for 30 minutes on ice. Following washes, a secondary antibody of 10 μg/mL goat anti-mouse-PE or 10 μg/mL goat anti-rabbit-PE was incubated with the cells for an additional thirty minutes on ice. Cells were washed to remove excess unbound antibody prior to analyzing cells on the flow cytometer. Non-transfected RBL cells did not stain positively with the mouse IgG3 isotype control (Fig. 5.6A), the anti-HA antibody (Fig. 5.6B), the rabbit IgG isotype control (Fig. 5.6C) or the rabbit anti-sCSF-1R antibody (Fig. 5.6D). RBL cells expressing the IpLITR/IpFcRγL fusion protein did not bind the mouse IgG3 isotype control (Fig. 5.7A). However, the IpLITR/IpFcRγL RBL cells were recognized by anti-HA IgG (Fig. 5.7B). The IpLITR/IpFcRγL expressing cells were not recognized by the rabbit IgG (Fig. 5.7C) or the anti-sCSF-1R IgG (Fig. 5.7D). These results suggest that the antibody to goldfish sCSF-1R does not bind to other proteins containing immunoglobulin domains.

Three clones of RBL cells, each expressing the sCSF-1R/IpFcRγL fusion protein, were generated. Each of the RBL cells expressing a different construct were not recognized by the mouse IgG3 isotype control (Fig. 5.8A, 5.9A, 5.10A), the anti-HA IgG3 (Fig. 5.8B, 5.9B, 5.10B), or the rabbit IgG isotype control (Fig. 5.8C, 5.9C, 5.10C). However, when cells from each RBL cell line expressing the different sCSF-1R/IpFcRγL constructs were incubated with anti-sCSF-1R IgG, a

positive population of CSF-1R$^+$ cells was observed, ranging from 19.6% - 49.5% positive depending on the particular construct used to transfect the RBL cells, compared to the SPF rabbit IgG isotype control stained cells (Fig 5.8D, 5.9D, 5.10D). Lastly, the sCSF-1R/IpFcRγL construct-1 and construct-3 were incubated with a 1:10 dilution of both the rabbit IgG isotype control and the anti-sCSF-1R antibody to demonstrate the ability of the binding to be titrated. Cells were incubated with 2.5 μg/mL rabbit IgG (Fig. 5.11A,C) or 2.5 μg/mL of anti-sCSF-1R IgG (Fig. 5.11B,D). For both construct-1 and construct-3, the CSF-1R positive population of cells disappeared (Fig. 5.11B, D, respectively). From these experiments, I believe the sCSF-1R antibody specifically recognizes the CSF-1R protein on the surface of the cells.

To further examine specificity of the sCSF-1R IgG antibody in fish systems, I performed immunostaining on two additional cell populations that should be negative for CSF-1R surface expression. The first cell population tested was a catfish B-cell line, 3B11. As expected, the 3B11 cell population was negative for CSF-1R (Fig. 5.12A), but did stain positively with the 9E1 hybridoma supernatant that recognizes catfish B-cell receptor, compared to the isotype control (Fig. 5.12B). Similarly, a goldfish fibroblast cell line, CCL71 cells, were also negative for surface CSF-1R when incubated with the anti-sCSF-1R antibody compared to the rabbit IgG isotype control (Fig 5.12C). I previously reported that purified goldfish kidney neutrophils were not bound by the anti-sCSF-1R antibody [33] (as discussed in chapter 7).

5.2.5 Distribution of CSF-1R$^+$ cells from goldfish tissues

To examine the presence of CSF-1R$^+$ cells in goldfish tissues, the kidney, spleen, liver and brain tissues were isolated from three individual fish, during two independent replicates, and pushed through a wire mesh screen to achieve a single cell suspension. Cells were layered over 51% Percoll and the cells at the interface collected, washed and used for immunostaining experiments. Cells isolated from the kidney, spleen, liver or brain were incubated with 25 μg/mL rabbit IgG or 25 μg/mL anti-sCSF-1R IgG for 30 minutes on ice, washed, incubated with 10 μg/mL goat anti-rabbit IgG-PE antibody for an additional thirty minutes on ice, and analyzed by flow cytometry. The populations of cells from the kidney (Fig. 5.13A), spleen (Fig. 5.13B), and liver (Fig. 5.13C) were positive for CSF-1R, whereas cells from brain tissue did not exhibit positive staining with the anti-sCSF-1R antibody, compared to the rabbit IgG isotype control (Fig. 5.13D). These data suggest that there are populations of CSF-1R$^+$ cells, indicative of cells from the macrophage lineage, present in the kidney, spleen, and liver, but not in the brain. These results coincide with studies performed using gilthead seabream, where CSF-1R$^+$ cells were observed in the kidney, spleen, liver, thymus and gill, and not in the brain or intestine [27]. However, the inability to detect a population of CSF-1R$^+$ cells from the goldfish brain is likely the result of the broad non-specificity of the isotype control, thereby precluding the identiication of a CSF-1R$^+$ population from the brain.

5.2.6 Analysis of CSF-1R$^+$ cells in primary kidney macrophage (PKM) cultures

The kidney is the major hematopoietic organ in teleosts and the Belosevic lab has previously established a primary kidney macrophage culture in which all three populations, progenitors, monocytes and macrophages, are present [28, 29]. Based on transcription factor mRNA levels, I proposed that the progenitor cell pool is a mixture of stem and progenitor cells from the goldfish kidney, and that the populations of stem/progenitor cells changed over time of culture [34] (the focus of chapter 6). Therefore, I wanted to assess whether I could identify a population of CSF-1R$^+$ progenitor cells, and to examine if this population of CSF-1R$^+$ cells changed over time of culture. In the first set of experiments, PKMs from day 0, day 1, day 2, day 3, or day 4 cultures were incubated with the anti-sCSF-1R antibody or the rabbit IgG isotype control. Following incubation with the secondary antibody, the cells were analyzed using flow cytometry based on size, internal complexity, and fluorescence. PKMs established from seven fish, during two independent experiments, were used, and a representative fish is shown.

Over the entire time period examined, there was a population of PKM cells that were positive for CSF-1R (Fig. 5.14). To determine which sub-populations of cells were responsible for the shift in fluorescence, cells were back-gated based on size and internal complexity. Three gates were used, the R1 gate consisting of small progenitors/lymphocytes, the R2 gate consisting of macrophages, and the R3 gate consisting of monocytes. Cells within the progenitor cell gate showed increased fluorescence, indicative of anti-CSF-1R IgG binding, suggestive of a population of CSF-1R$^+$ cells (Fig 5.15). The average of the ratio of median fluorescence intensity (MFI) of the cells incubated with the

anti-sCSF-1R IgG to the cells incubated with the isotype control was calculated for each time point (Table 5.1). Based on these calculations, there was a generalized decrease in the ratio of MFI of cells positive for CSF-1R in the progenitor cell gate over time (Fig. 5.15; Table 5.1). In particular, there was a significant decrease in the MFI progenitor cells at day 1, day 2, day 3 and day 4 compared to the day 0 progenitor cells (Table 5.1). Upon analyzing cells in the monocyte gate, cells incubated with the anti-sCSF-1R antibody had higher fluorescence intensity than progenitor cells and monocytes were more uniform in their binding by the sCSF-1R antibody. These data suggest the monocytes have more, and a consistent number of, CSF-1Rs on their surface (Fig. 5.16; Table 5.1). The decrease in MFI ratios of cells in the monocyte gate over time of culture (Table 5.1) can be attributed to the movement of these monocytes from the R3 gate towards the R2 gate. It should be noted that these cells always stained positively for sCSF-1R with a high intensity (not shown). The small number of cells within the R2 macrophage gate also appeared to bind, although with lower intensity, the sCSF-1R antibody (Fig. 5.17; Table 5.1). In this particular experiment, due to the early time period assessed, the cells in the culture did not have sufficient time to develop into macrophages. However, previous studies in the laboratory have shown that mature macrophages are recognized by CSF-1R antibody with a similar intensity as monocytes [26].

The decrease in progenitor cells bound by the sCSF-1R antibody suggested two possible outcomes; (1) the decrease in CSF-1R$^+$ cells was due to the binding of the receptor by endogenous CSF-1 produced by the PKMs in culture, thus blocking the CSF-1R antibody sites on the membrane CSF-1R on progenitors, or (2) the CSF-1R$^+$ progenitor cells are a finite pool of progenitors

and are not capable of replenishing their numbers as the macrophage development progresses, such that the CSF-1R$^+$ progenitors are differentiating into monocytes and macrophages thus leaving the R1 progenitor cell gate. To address the potential binding of endogenous CSF-1 to the membrane-bound CSF-1R on progenitor cells, an acid treatment was used to remove any non-covalently bound molecules from the surface of the cells. Following PBS or acid treatment, PKMs were incubated with the isotype control or the sCSF-1R antibody, followed by secondary antibody, and analyzed using flow cytometry as in the first experiment. Acid treatment was performed on day 0 and day 4 PKMs. The ratio of MFI was calculated in a similar manner (Table 5.2). When the MFI ratios of untreated or acid-treated progenitor cells were compared on both day 0 and day 4, there were no significant differences. These data suggest that the decrease in CSF-1R$^+$ progenitor cells from day 0 to day 4 is not the result of endogenous CSF-1 binding to membrane CSF-1R to prevent anti-sCSF-1R antibody recognition. A similar trend was observed with macrophages at day 0 and day 4 (Table 5.2). Inexplicably, when cells within the monocyte gate at day 0 were analyzed, a significant decrease in the MFI ratio of acid-treated cells compared to the non-treated cells was observed (Table 5.2). However, when the population of untreated versus acid treated monocytes from day 4 PKM cultures were compared, no significant difference was observed in MFI (Table 5.2).

5.3 Discussion

The focus of this chapter was to examine the ability of antibodies to goldfish PROMININ and CSF-1R to recognize a population(s) of progenitor cells.

In the first part of this chapter, a recombinant protein corresponding to the first extracellular loop of PROMININ (rgPECL-1) was generated and a polyclonal anti-rgPECL-1 antibody produced. While the anti-rgPECL-1 antibody was capable of recognizing the rgPECL-1 protein in a western blot and was useful in immunoprecipitating the rgPECL-1 protein from solution, the anti-rgPECL-1 was limited in recognizing the native PROMININ protein. The anti-rgPECL-1 antibody recognized a protein of approximately 89 kDa, which is the predicted size of the native goldfish PROMININ molecule in the absence of glycosylations, but was unable to recognize any isolated membrane proteins. I believe the lack of anti-rgPECL-1 antibody recognition to be due to the extensive glycosylation that occurs on the two large extracellular loops of the native PROMININ molecule. The presence of glycosylations on PROMININ would most likely obscure the protein epitopes that the rgPECL-1 antibody was generated against. However, based on the ability of the anti-rgPECL-1 antibody to specifically recognize rgPECL-1 from bacterial cell lysates, it would seem that this antibody has excellent potential to be used for identifying native PROMININ in the absence of glycosylations. Therefore, procedures to deglycosylate membrane proteins on the surface of cells could be optimized to allow for anti-rgPECL-1 recognition of a population of progenitor cells expressing PROMININ. If deglycosylation procedures are optimized correctly, the anti-rgPECL-1 antibody may be used to determine if there are sub-populations of progenitor cells expressing PROMININ, and what percentage of progenitors makes up this PROMININ^{+} population over time of cultivation. Although, it is likely that another antibody to a recombinant PROMININ molecule, produced in a mammalian system to allow for

glycosylation, will be required for recognition and isolation of viable progenitor cell populations.

In the second part of this chapter, I presented evidence to attest to the specificity of the anti-sCSF-1R antibody to goldfish CSF-1R. The anti-CSF-1R antibody recognized goldfish CSF-1R on the surface of transfected RBL cells, but did not recognize a two-immunoglobulin domain catfish LITR protein on the surface of RBLs. Although the anti-sCSF-1R antibody recognized CSF-1R on the surface of sCSF-1R/IpFcRγL transfected RBLs, there was variability in the percentage of positive cells expressing CSF-1R. I believe this to be due to the differing transfection efficiencies of the RBLs. Furthermore, the anti-sCSF-1R antibody did not recognize non-specific surface proteins of fish cells, as shown by the negative immunostaining of catfish B-cells and goldfish fibroblast cells with the anti-sCSF-1R antibody. Taken together, these results demonstrate the specificity of the anti-sCSF-1R antibody to CSF-1R.

Consistent with a study by Mulero *et al*. [27] examining macrophage distribution in gilthead seabream tissues using a monospecific anti-CSF-1R antibody, CSF-1R$^+$ cells were observed from goldfish kidney, spleen, and liver tissues. The CSF-1R$^+$ cells from goldfish tissues may represent populations of tissue differentiated macrophages, or possibly their precursors as observed in mammalian systems, such as the Kuppfer cells of the liver, reviewed in [35, 36]. However, I was unable to conclusively observe a population of CSF-1R$^+$ cells from the brain. The inability to identify a population of CSF-1R$^+$ cells from the brain may be due to the non-specific binding of the isotype control to brain cells. Studies in mammalian systems have demonstrated that macrophages reside in the brain as microglia and that primitive macrophages developed during

embryogenesis gives rise to microglia in adults [37]. The development of microglia from primitive macrophages is dependent on signaling through CSF-1R, likely through binding of IL-34, and CSF-1R expression is retained on the brain microglia [37]. In zebrafish, the movement of macrophages into the brain is a CSF-1R dependent process [23]. Microglia from goldfish brain tissue have been identified and shown to be capable of phagocytosis [38]. However, while it seems likely that CSF-1R would be involved in goldfish microglia development, these studies have not yet been performed.

The expression of CSF-1R mRNA and protein has been associated with cells of the mononuclear phagocyte lineage of the hematopoietic cells in the mouse model system [39, 40], although low levels of CSF-1R mRNA are expressed in mammalian neutrophils [41]. In teleost model systems, studies have shown mRNA expression of CSF-1R in macrophages and monocytes [23, 25, 26, 42] and their progenitors [43]. However limited studies have been performed in parallel using antibodies to teleost CSF-1R [27].

In this chapter, I showed that anti-goldfish sCSF-1R antibody specifically recognized a population of goldfish kidney progenitor cells, along with monocytes, macrophages. Freshly isolated kidney progenitor cells exhibited the greatest level of CSF-1R on their surface, however, CSF-1R expression decreased in the progenitor cell population coinciding with the production of monocytes and macrophage *in vitro*. Studies in our laboratory have previously shown that the PKMs cells are capable of producing their own endogenous growth factors, of which CSF-1 is believed to be one of them [31, 44]. Therefore, it was possible that the decrease in CSF-1R expression on the surface of the progenitor cells may have been due to the binding of CSF-1 to CSF-1R, thereby

preventing the anti-sCSF-1R antibody from binding. However, upon performing brief acid treatment of the cells to dissociate any ligand bound to surface receptors, I did not observe a significant change in the MFI ratio, indicating the anti-sCSF-1R antibody was not being blocked by endogenous ligand bound to the CSF-1R on the surface of any of the mononuclear phagocyte populations. However, it is possible that rapid CSF-1R internalization could be taking place due to the presence of endogenous ligand [15]. These data could indicate a decrease in the number of macrophage committed progenitor cells, a decrease in the number of receptors on the surface of the macrophage progenitors, or a combination of the two. Regardless, I believe the expression of CSF-1R on progenitor cells to suggest the commitment of these progenitors to the macrophage lineage.

Although other antibodies have been developed that recognize teleost macrophages [45, 46], the rabbit-anti-goldfish sCSF-1R was the first antibody developed to fish CSF-1R that recognizes a population of CSF-1R$^+$ progenitor cells. The ability of the anti-goldfish sCSF-1R antibody to recognize native CSF-1R on the surface of cells has the potential for use in identifying and isolating live CSF-1R$^+$ cells (macrophages, monocytes and their progenitors) for use in future functional assays or for use in understanding lineage fate decisions of fish progenitor cells in vitro. To my knowledge, I am the first to report on the expression of CSF-1R on teleost macrophage committed progenitors, with CSF-1R expression increasing with macrophage development.

Table 5.1: Median fluorescent intensity (MFI) ratios of PKM cell populations incubated with rabbit anti-sCSF-1R IgG over time of culture.

	Progenitors (R1 cells)	Monocytes (R3 cells)	Macrophages (R2 cells)
Day 0	10.05 ± 1.71	30.26 ± 2.95	5.29 ± 0.97
Day 1	3.81 ± 0.62*	16.52 ± 4.16*	2.68 ± 0.43*
Day 2	3.45 ± 0.24*	8.44 ± 1.33*	3.25 ± 0.43
Day 3	2.29 ± 0.16*	3.39 ± 1.06*+	2.41 ± 0.51*
Day 4	2.14 ± 0.25*	1.78 ± 0.338*+	2.21 ± 0.29*

* denotes a significant difference from day 0, + denotes a significant difference from day 1, $P < 0.05$. One-way ANOVA with a Tukey's multiple comparison test.

Table 5.2: Median fluorescent intensity (MFI) ratios of PKM cell populations treated with acid at day 0 and day 4 of culture.

		Progenitors (R1 cells)	Monocytes (R3 cells)	Macrophages (R2 cells)
Day 0	Untreated	4.63 ±1.25	23.13 ± 4.07	3.30 ± 0.73
	Acid-treated	2.86 ± 0.73	14.53 ± 2.46*	2.94 ± 0.33
Day 4	Untreated	2.11 ± 0.40	2.37 ± 0.64	2.53 ± 0.31
	Acid-treated	2.34 ± 0.36	2.79 ± 0.79	2.93 ± 0.23

* denotes a significant difference from the untreated cell population, $P < 0.05$.

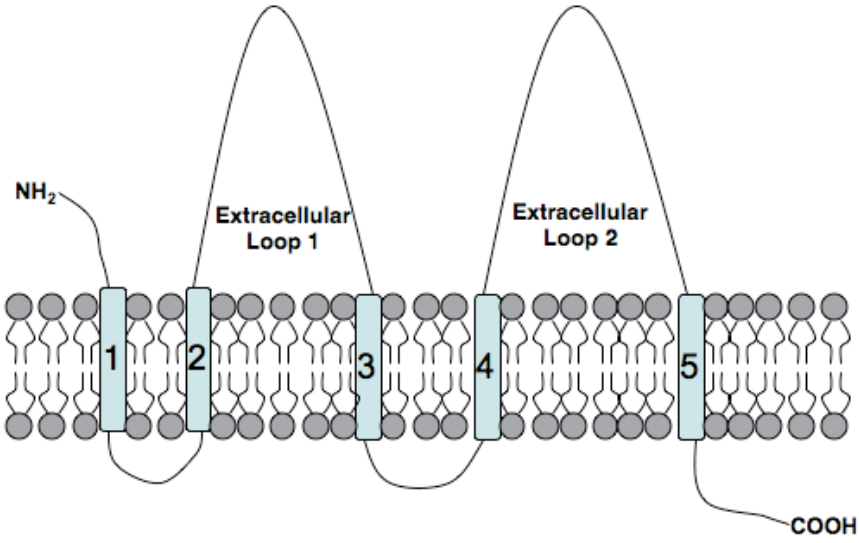


Figure 5.1: Schematic diagram of goldfish PROMININ.
Goldfish PROMININ is a pentaspan transmembrane protein with an extracellular amino terminus, two large extracellular loops that have ten potential N-linked glycosylation sites, and an intracellular carboxy tail. The prominin signature sequence is located in extracellular loop 2, proximal to the fifth transmembrane region.

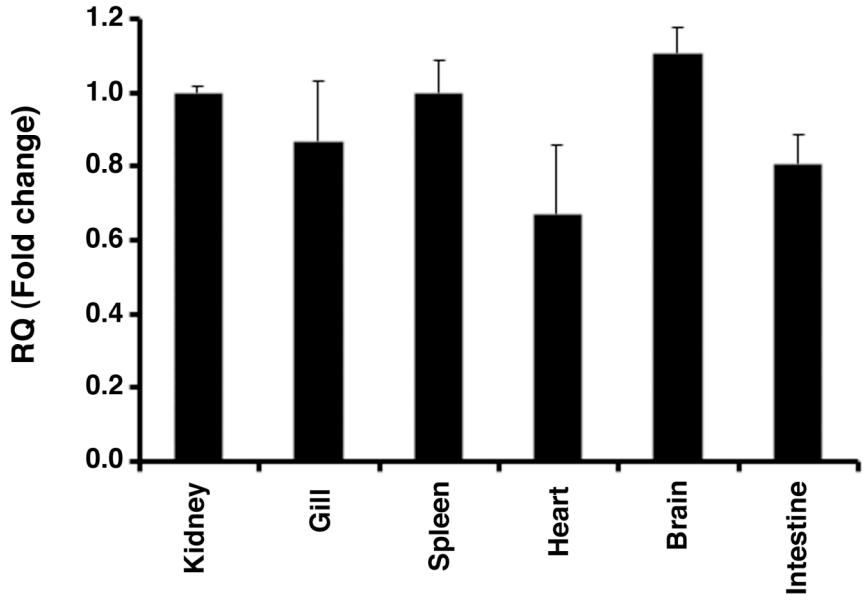


Figure 5.2: Expression of *prominin* in goldfish tissues as determined by quantitative PCR.
The mRNA levels of goldfish *prominin* in tissues from four fish were determined through quantitative PCR. The mRNA levels of prominin were normalized to those in kidney as the reference tissue (n = 4).

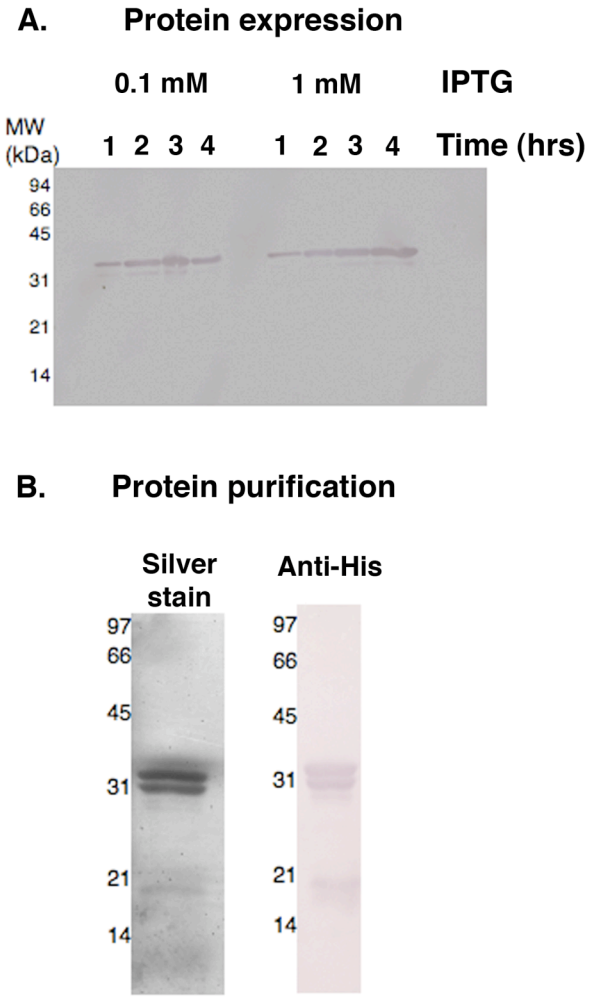


Figure 5.3: Protein expression and purification of recombinant goldfish PROMININ extracellular loop 1 (rgPECL-1).
The nucleic acid sequence encoding for the first PROMININ extracellular loop was cloned into a pET151 expression vector and transformed into BL21star *E. coli*. Bacteria were induced with 0.1 mM or 1 mM IPTG to induce protein expression and the presence of protein determined using western blotting with an anti-his antibody after 1 hr, 2 hrs, 3 hrs, or 4 hrs post induction (A). rgPECL-1 was subsequently purified using MagneHis beads and the purified protein run on a 12% SDS-PAGE gel and silver stained (B). A duplicate gel was transferred to a nitrocellulose membrane and blotted with an anti-His and goat anti-mouse antibody to confirm the presence of recombinant protein (B). The three bands were excised from the gel and submitted for mass spectrometry to confirm they were rgPECL-1.

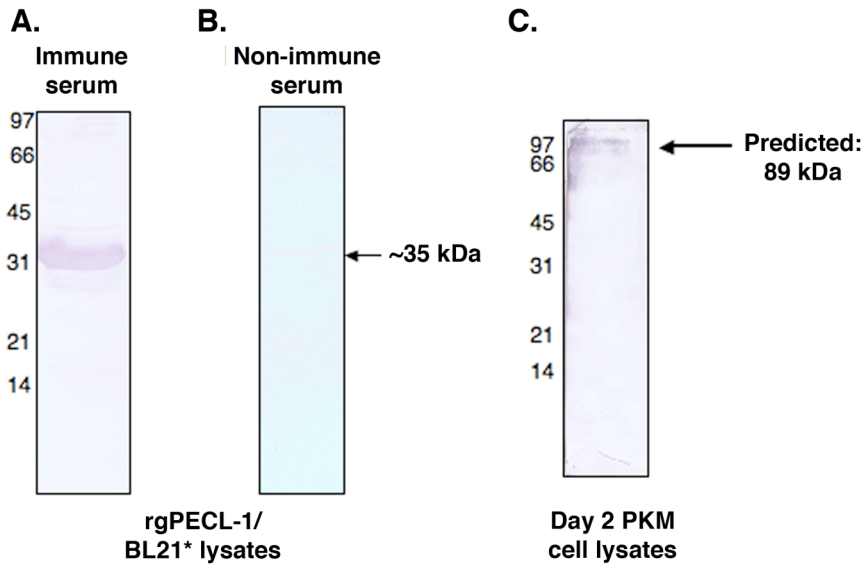


Figure 5.4: Detection of rgPECL-1 and the potential native protein using an anti-rgPECL-1 antibody.
Bacterial lysates from BL21star E. coli expressing rgPECL-1 were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blotted with a 1:5000 dilution of anti-rgPECL-1 serum (A) or a 1:5000 dilution of non-immune serum from a pre-immunization bleed (B). The anti-rgPECL-1 serum was used at a 1:1000 dilution to detect the native PROMININ protein in day 2 PKM cell lysates (C).

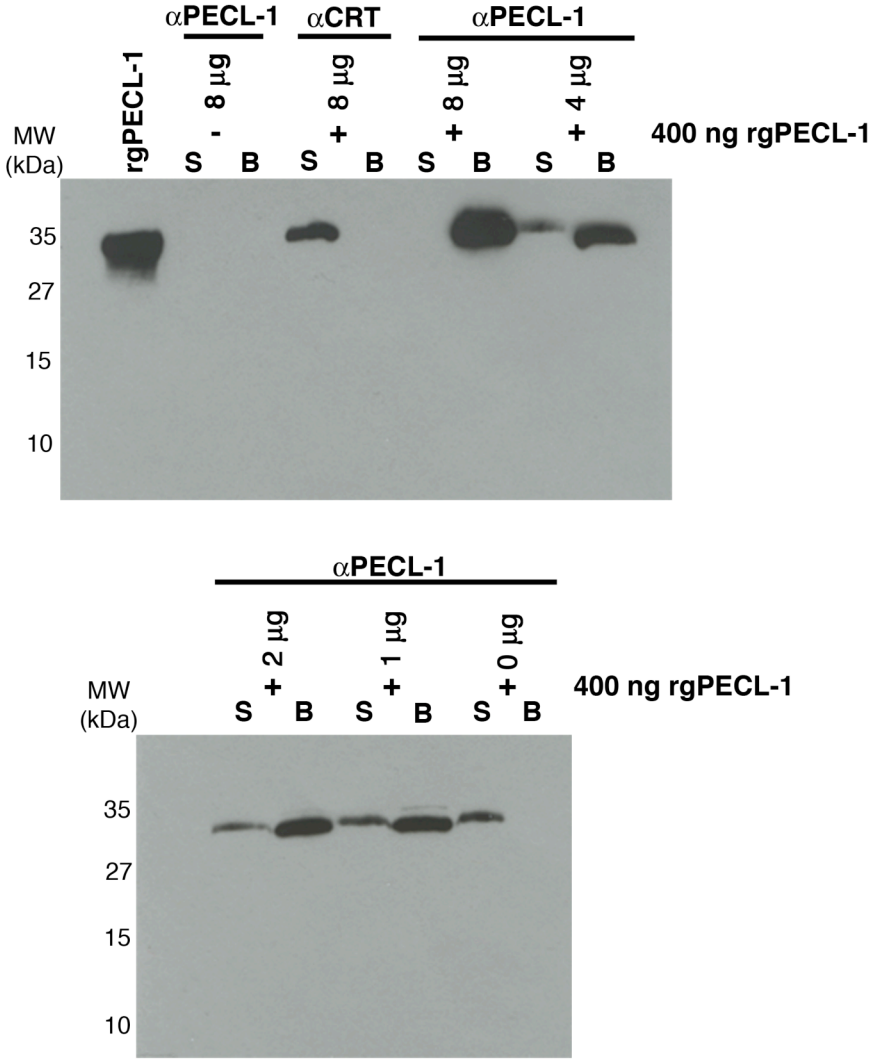


Figure 5.5: Immunoprecipitation of rgPECL-1 by the anti-rgPECL-1 antibody.
Recombinant goldfish PECL-1 (400 ng) was incubated with 8 μg of anti-CRT (negative control) or with varying concentrations of anti-rgPECL-1 antibody. Following overnight incubation, sepharose G beads were added and incubated for an additional hour. Supernatants (S) were collected, and beads washed before they were boiled in SDS-PAGE loading buffer (B). Supernatant (S) and bead (B) fractions were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were probed with an anti-V5-HRP antibody to detect the presence of rgPECL-1.

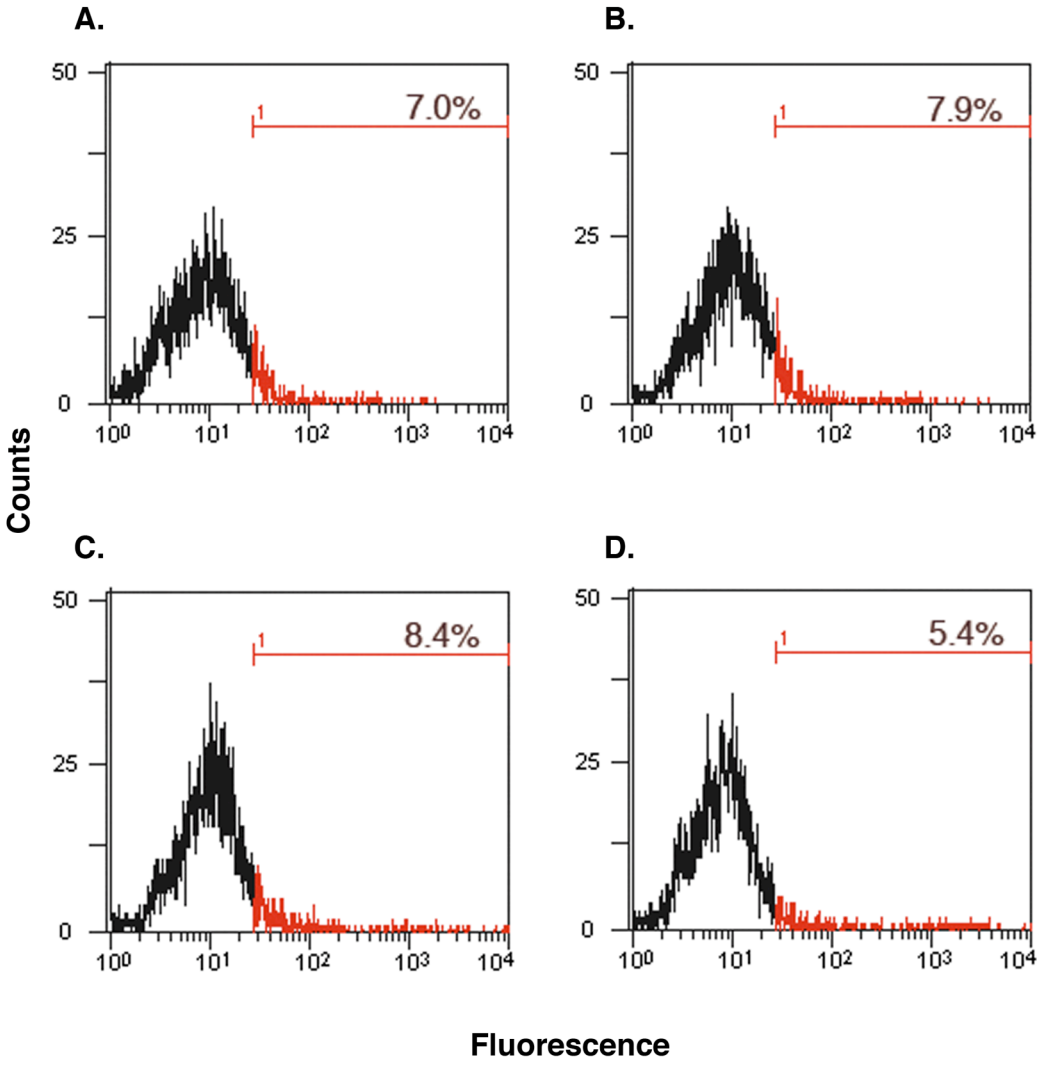


Figure 5.6: Immunofluorescence of non-transfected RBL 2H3 cells. Non-transfected RBL cells were incubated with 20 μg/mL mouse IgG3 isotype control (A), 20 μg/mL mouse anti-HA IgG3 (B), 25 μg/mL rabbit IgG isotype control (C), or 25 μg/mL rabbit anti-sCSF-1R IgG antibody (D), followed by a secondary antibody of either 10 μg/mL goat anti-mouse IgG-PE (A and B) or 10 μg/mL goat anti-rabbit IgG-PE (C and D). A total of10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the IgG3 isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

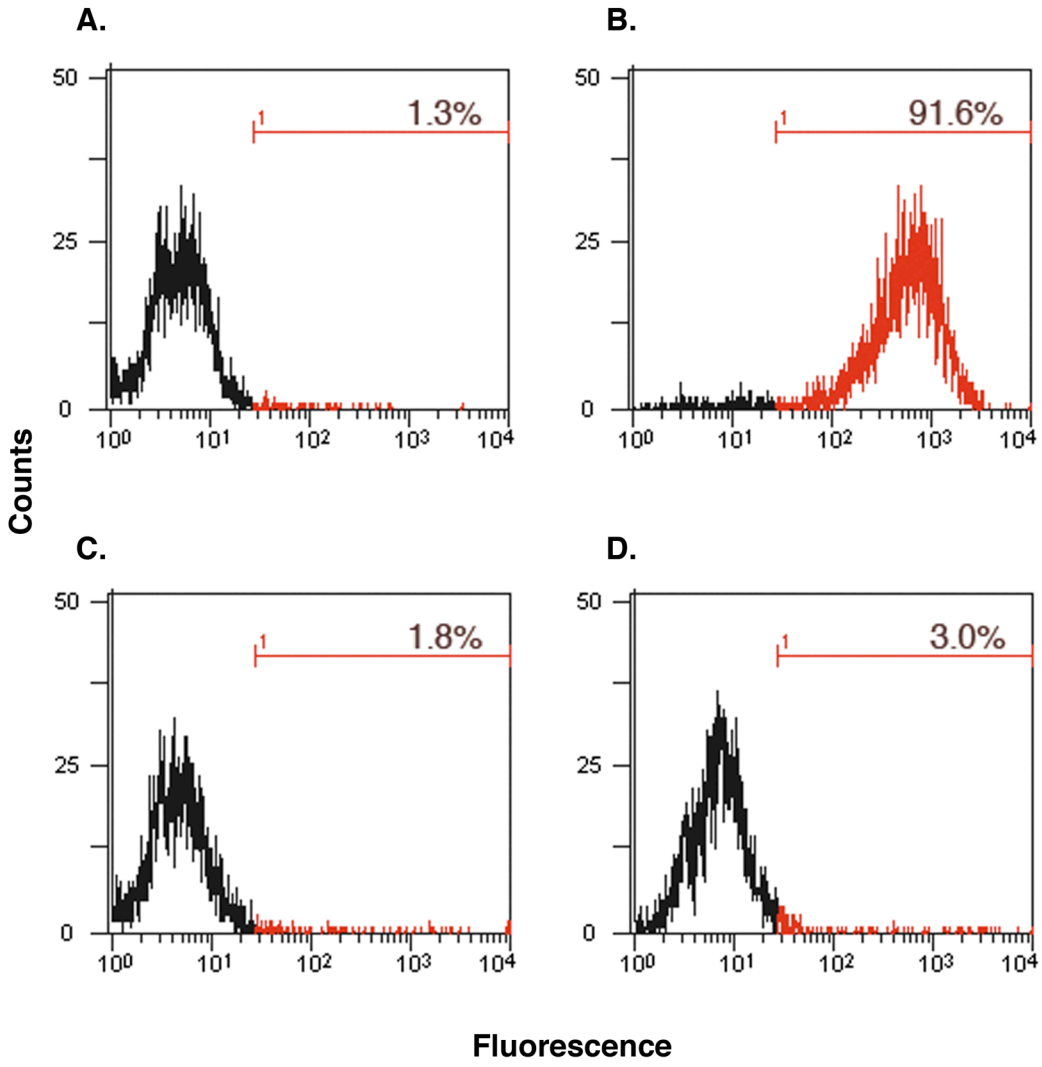


Figure 5.7: Immunofluorescence of IpLITR/IpFcRγL transfected RBL2H3 cells.
IpLITR/IpFcRγL transfected RBL cells were incubated with 20 μg/mL mouse IgG3 isotype control (A), 20 μg/mL mouse anti-HA IgG3 (B), 25 μg/mL rabbit IgG isotype control (C), or 25 μg/mL rabbit anti-sCSF-1R IgG antibody (D), followed by a secondary antibody of either 10 μg/mL goat anti-mouse IgG-PE (A and B) or 10 μg/mL goat anti-rabbit IgG-PE (C and D). 10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the IgG3 isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

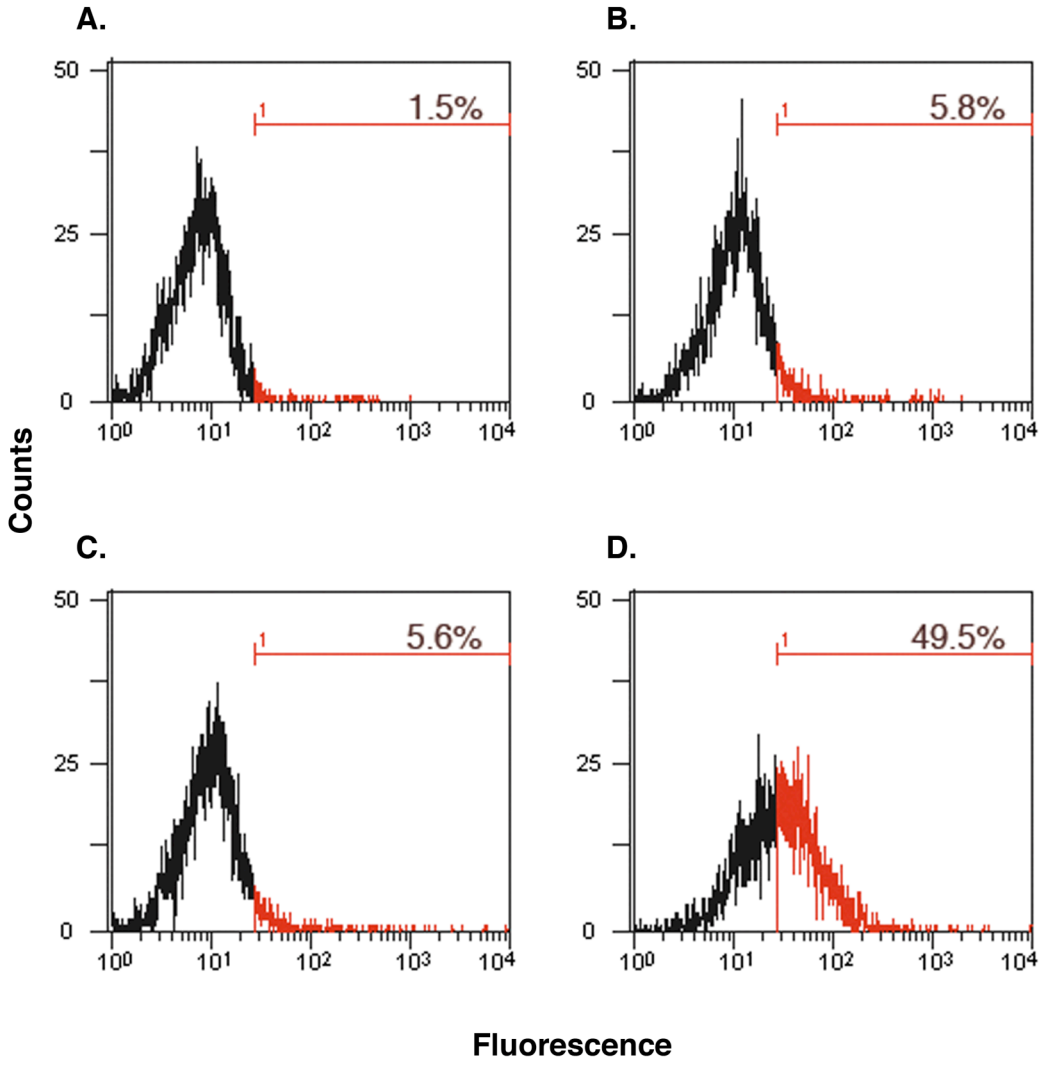


Figure 5.8: Immunofluorescence of sCSF-1R/IpFcRγL, construct-1 transfected RBL2H3 cells.
sCSF-1R/IpFcRγL transfected RBL2H3 cells were incubated with 20 μg/mL mouse IgG3 isotype control (A), 20 μg/mL mouse anti-HA IgG3 (B), 25 μg/mL rabbit IgG isotype control (C), or 25 μg/mL rabbit anti-sCSF-1R IgG antibody (D), followed by a secondary antibody of either 10 μg/mL goat anti-mouse IgG-PE (A and B) or 10 μg/mL goat anti-rabbit IgG-PE (C and D). 10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the IgG3 isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

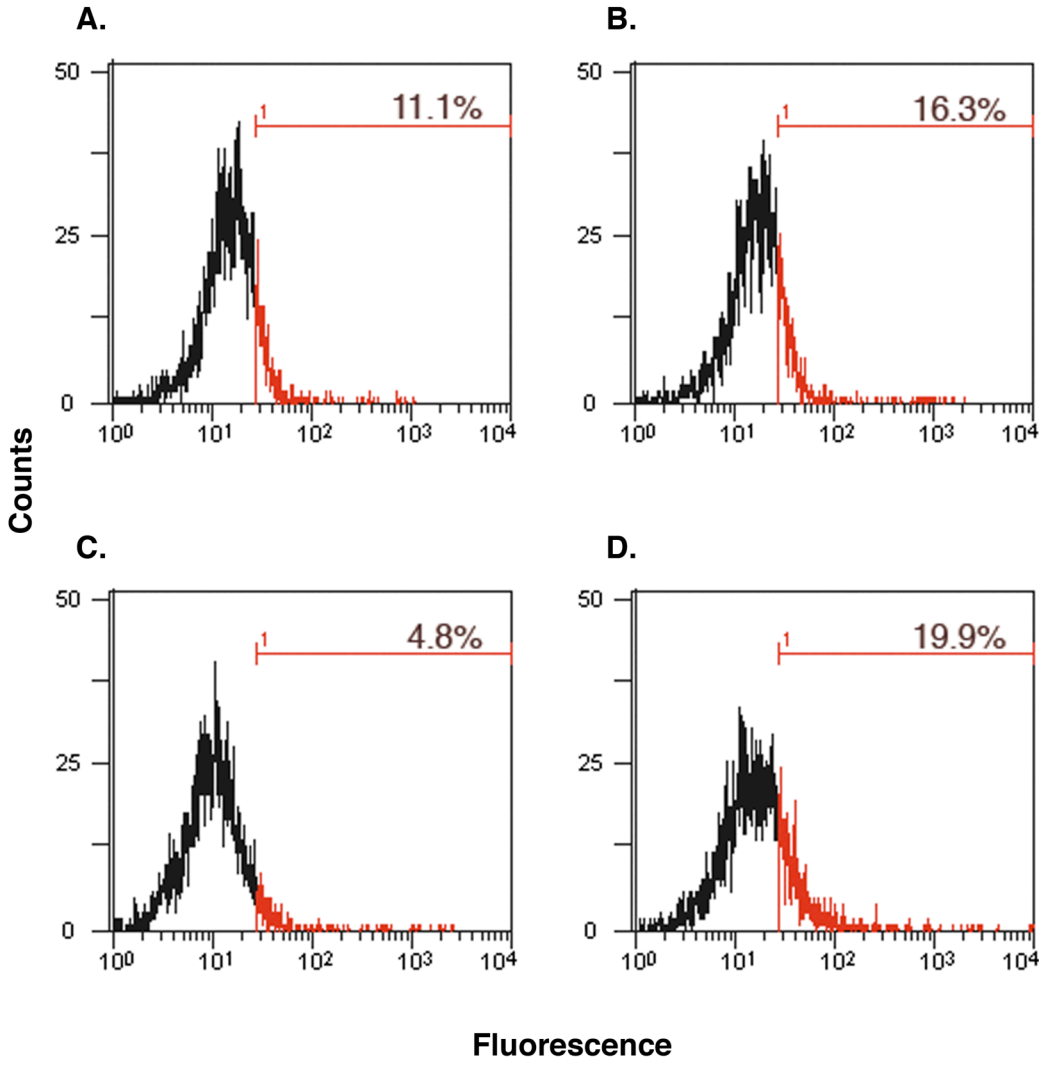


Figure 5.9: Immunofluorescence of sCSF-1R/IpFcRγL, construct-2 transfected RBL2H3 cells.
sCSF-1R/IpFcRγL transfected RBL2H3 cells were incubated with 20 μg/mL mouse IgG3 isotype control (A), 20 μg/mL mouse anti-HA IgG3 (B), 25 μg/mL rabbit IgG isotype control (C), or 25 μg/mL rabbit anti-sCSF-1R IgG antibody (D), followed by a secondary antibody of either 10 μg/mL goat anti-mouse IgG-PE (A and B) or 10 μg/mL goat anti-rabbit IgG-PE (C and D). 10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the IgG3 isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

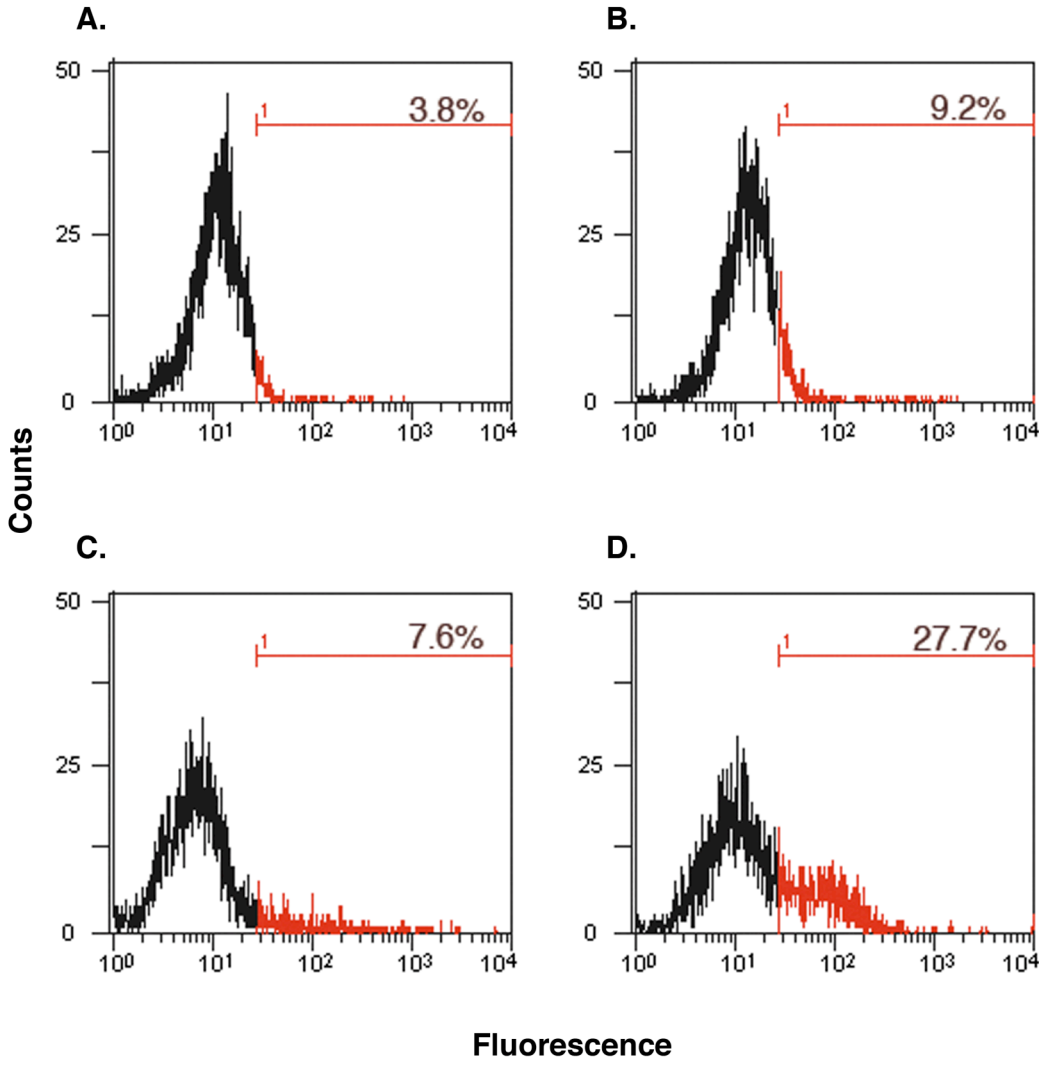


Figure 5.10: Immunofluorescence of sCSF-1R/IpFcRγL, construct-3 transfected RBL2H3 cells.
sCSF-1R/IpFcRγL transfected RBL2H3 cells were incubated with 20 μg/mL mouse IgG3 isotype control (A), 20 μg/mL mouse anti-HA IgG3 (B), 25 μg/mL rabbit IgG isotype control (C), or 25 μg/mL rabbit anti-sCSF-1R IgG antibody (D), followed by a secondary antibody of either 10 μg/mL goat anti-mouse IgG-PE (A and B) or 10 μg/mL goat anti-rabbit IgG-PE (C and D). 10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the IgG3 isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

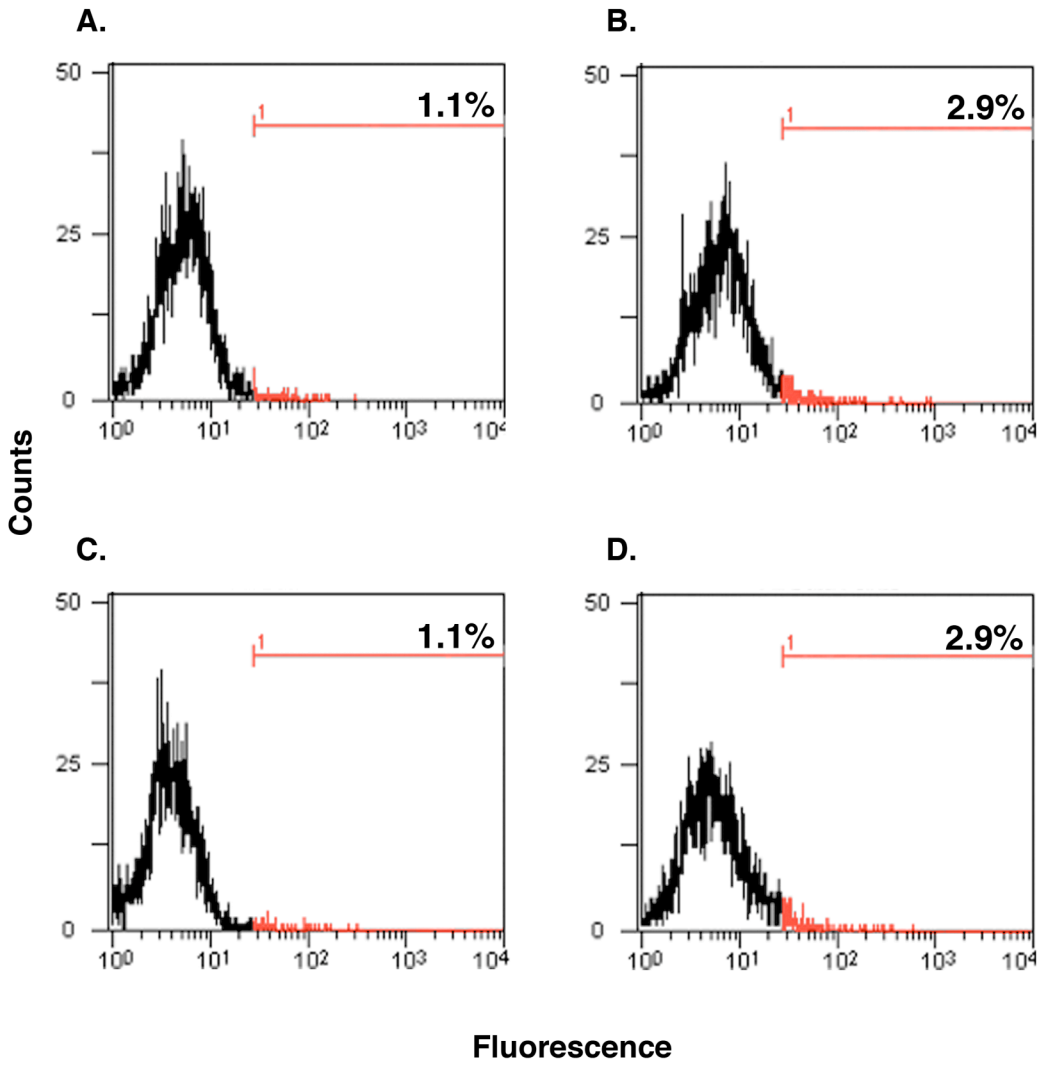


Figure 5.11: Immunofluorescence of sCSF-1R/IpFcRγL, construct-1 and construct-3 transfected RBL2H3 cells.
sCSF-1R/IpFcRγL transfected RBL2H3 cells, construct-1 (A and B) and construct-3 (C and D) were incubated with 2.5 μg/mL rabbit IgG isotype control (A and C), or 2.5 μg/mL rabbit anti-sCSF-1R IgG antibody (B and D), followed by a secondary antibody 10 μg/mL goat anti-rabbit IgG-PE. 10,000 events were analyzed by flow cytometry. Gate 1 was set up based on the isotype control to determine percent of immunofluorescent positive cells. Shown is a representative experiment of two that were performed.

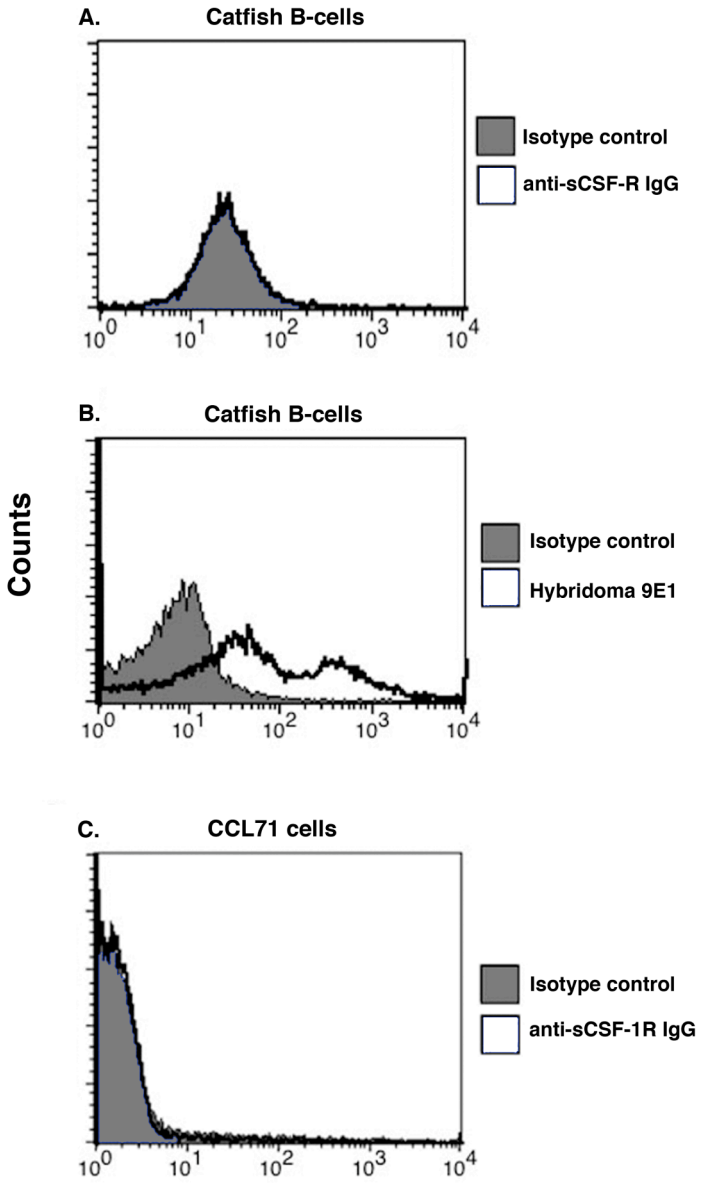


Figure 5.12: Immunofluorescent labeling of catfish 3B11 B-cells or goldfish CCL71 fin fibroblast cells with anti-sCSF-1R IgG.
(A) Catfish 3B11 cells were incubated with rabbit IgG isotype control or rabbit anti-sCSF-1R IgG. (B) Catfish 3B11 cells were incubated with mouse hybridoma isotype control or 7E9 mouse hybridoma supernatant that recognizes catfish IgM. (C) Goldfish CCL71 fin fibroblast cells were incubated with rabbit IgG isotype control or anti-sCSF-1R IgG antibody. 10 μg/mL of anti-mouse IgG-PE (B) or 10 μg/mL of anti-rabbit IgG-PE was used as the secondary antibody (A and C). 10,000 events were analyzed by flow cytometry.

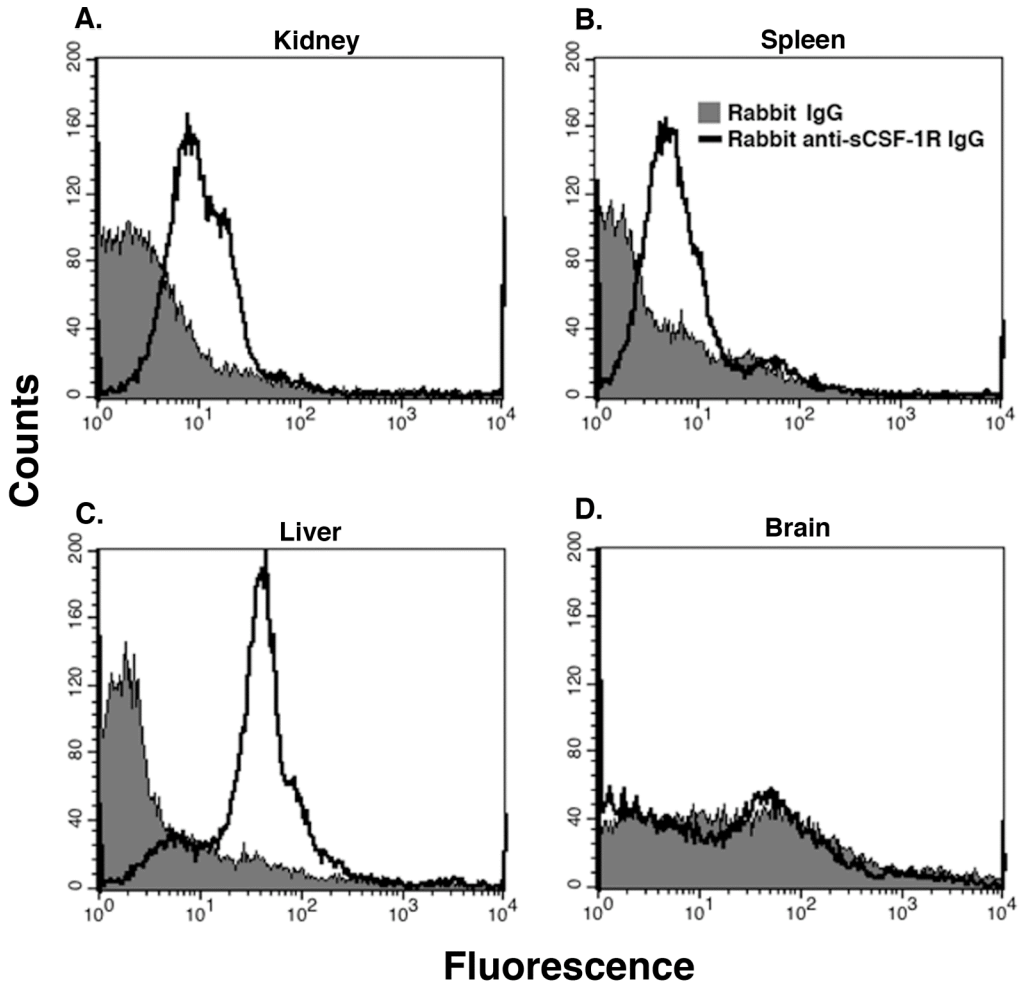


Figure 5.13: Immunofluorescence of goldfish mononuclear cells isolated from tissues.
Mononuclear cells from the (A) kidney, (B) spleen, (C) liver or (D) brain were incubated with 25 μg/mL rabbit IgG (isotype control) or anti-sCSF-1R. A secondary anti-rabbit IgG-PE antibody at a concentration of 10 μg/mL was used. 25,000 events were analyzed by flow cytometry.

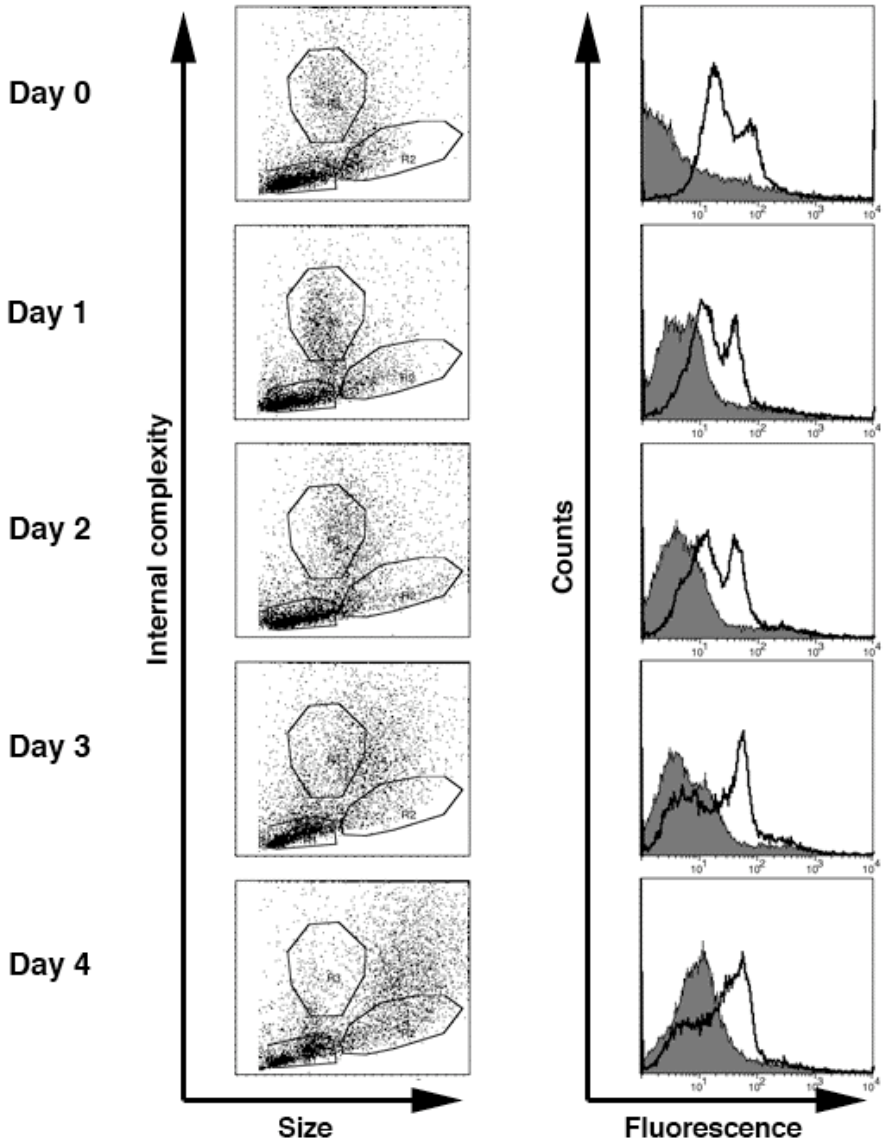


Figure 5.14: Immunofluorescence labeling of goldfish primary kidney macrophage (PKM) cells with anti-sCSF-1R IgG.
PKM cells from day 0 to day 4 of cultivation were incubated with 25 μg/mL rabbit IgG (grey shaded) or 25 μg/mL anti-sCSF-1R IgG (black line, unfilled). 10,000 events were analyzed based on internal complexity (side scatter), size (forward scatter) and fluorescence on FL2. Fluorescence of all cells within the culture was analyzed (whole culture). Shown here is a representative culture of cells established from an individual fish out of seven cultures examined.

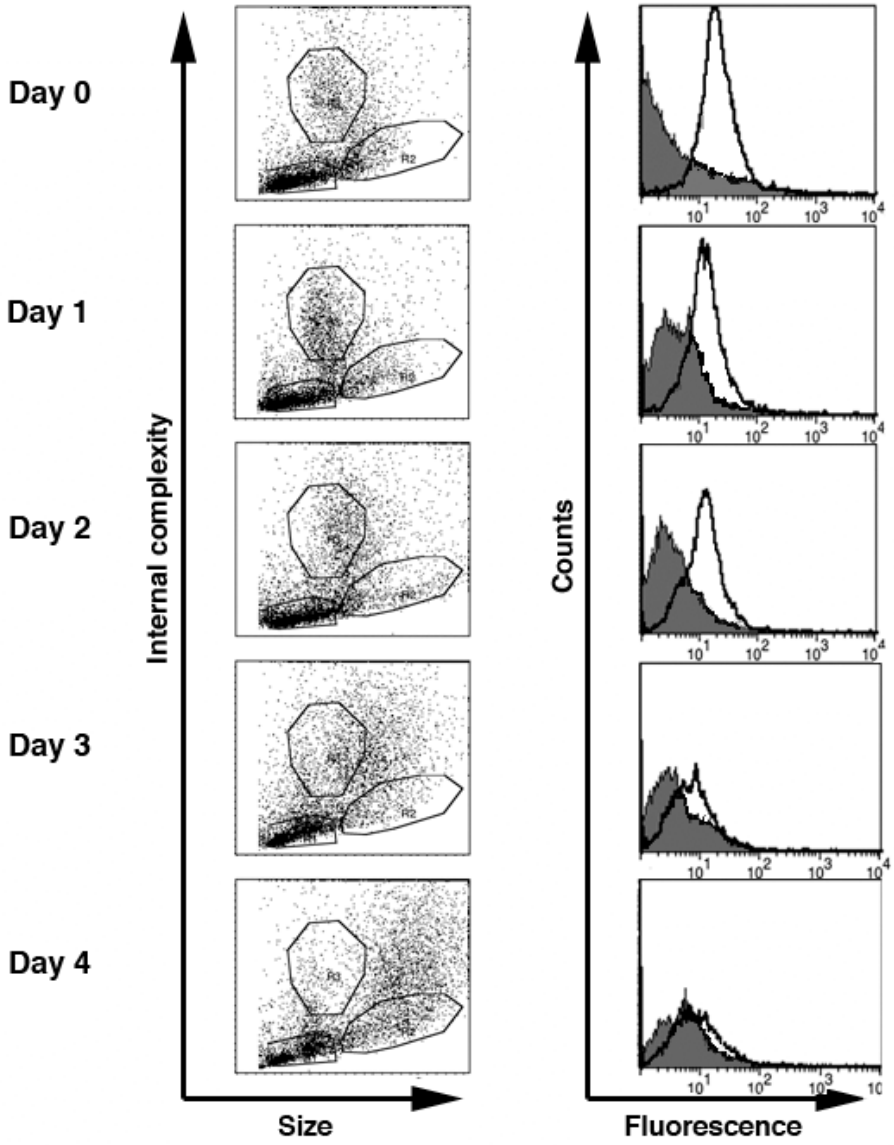


Figure 5.15: Immunofluorescence labeling of progenitor cells from goldfish primary kidney macrophage cultures.
Cells from the PKM cultures were incubated with 25 μg/mL rabbit IgG (grey shaded) or 25 μg/mL anti-sCSF-1R IgG (black line, unfilled) on day 0 to day 4 of cultivation. 10 μg/mL of anti-rabbit IgG-PE was used as the secondary antibody. 10,000 events were analyzed based on internal complexity (side scatter), size (forward scatter) and fluorescence on FL2. Fluorescence of all cells with the culture was analyzed (whole culture) and back gated based on the progenitor cell gate (R1). Shown here is a representative culture of cells established from an individual fish out of seven cultures examined.

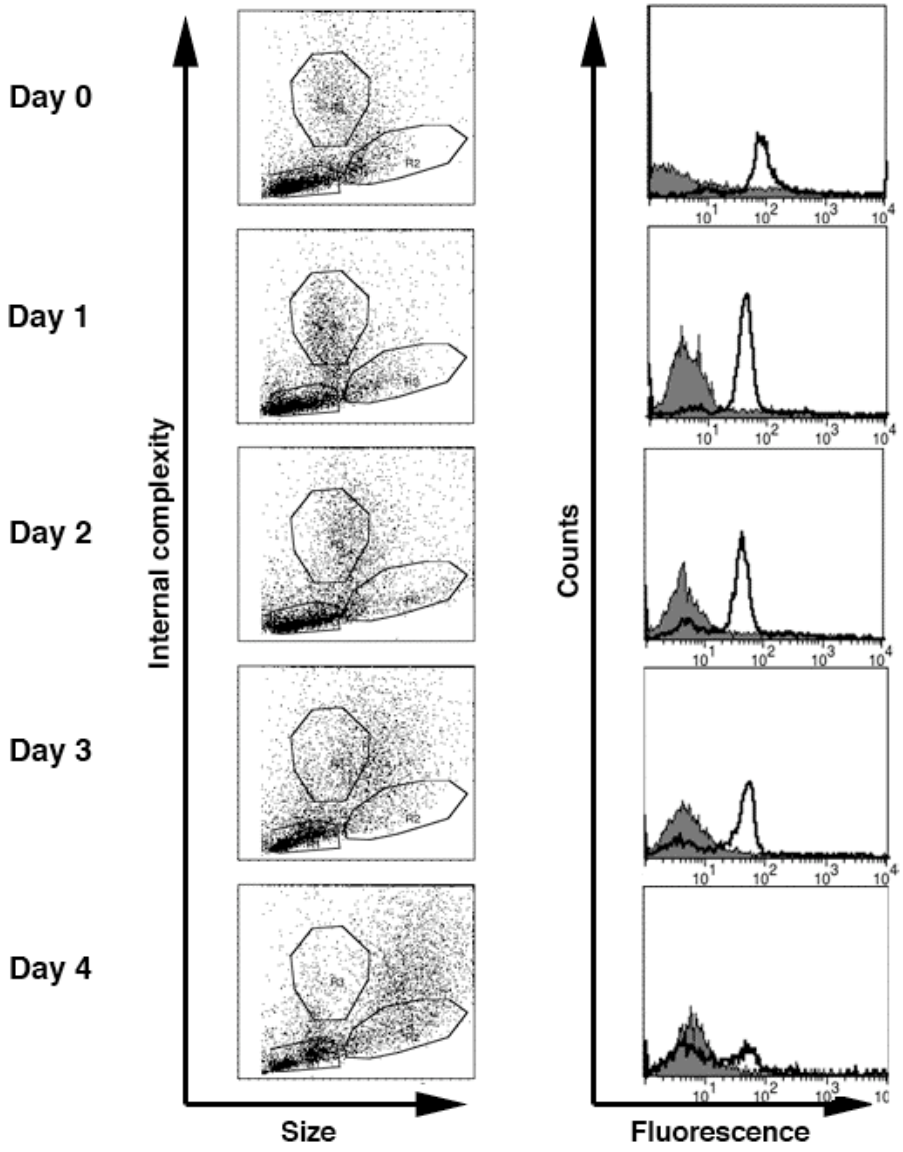


Figure 5.16: Immunofluorescence labeling of monocytes from goldfish primary kidney macrophage cultures.
Cells from the PKM cultures were incubated with 25 μg/mL rabbit IgG (grey shaded) or 25 μg/mL anti-sCSF-1R IgG (black line, unfilled) on day 0 to day 4 of cultivation. 10 μg/mL of anti-rabbit IgG-PE was used as the secondary antibody. 10,000 events were analyzed based on internal complexity (side scatter), size (forward scatter) and fluorescence on FL2. Fluorescence of all cells with the culture was analyzed (whole culture) and back gated based on monocyte gate (R3). Shown here is a representative culture of cells established from an individual fish out of seven cultures examined.

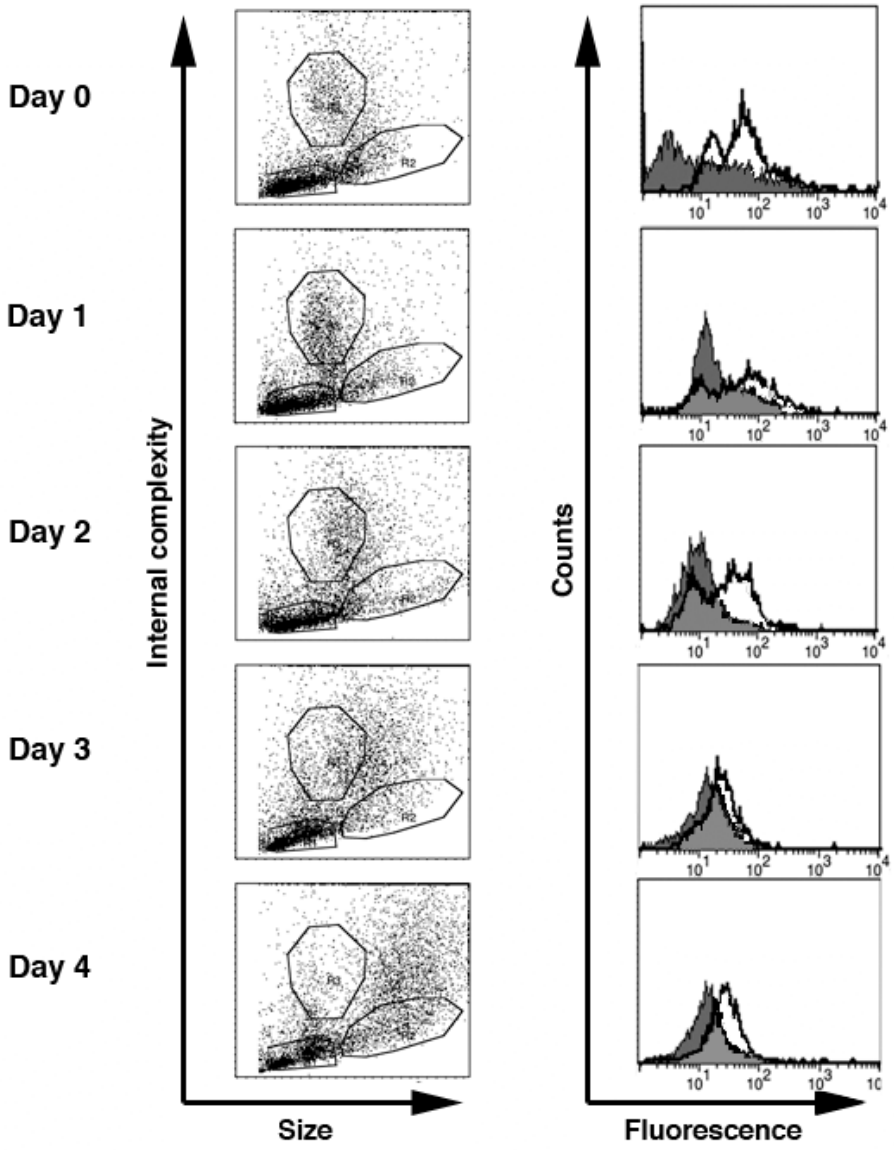


Figure 5.17: Immunofluorescence labeling of macrophages from goldfish primary kidney macrophage cultures.
Cells from the PKM cultures were incubated with 25 *μ*g/mL rabbit IgG (grey shaded) or 25 *μ*g/mL anti-sCSF-1R IgG (black line, unfilled) on day 0 to day 4 of cultivation. 10 *μ*g/mL of anti-rabbit IgG-PE was used as the secondary antibody. 10,000 events were analyzed based on internal complexity (side scatter), size (forward scatter) and fluorescence on FL2. Fluorescence of all cells with the culture was analyzed (whole culture) and back gated based on the macrophage cell gate (R2). Shown here is a representative culture of cells established from an individual fish out of seven cultures examined.

5.4 References

[1] **Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al.** AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90(12):5002-12.

[2] **Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, et al.** A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood. 1997;90(12):5013-21.

[3] **Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, et al.** The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem. 2000;275(8):5512-20.

[4] **Corbeil D, Roper K, Hannah MJ, Hellwig A, Huttner WB.** Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells - a combination of apical sorting and retention in plasma membrane protrusions. J Cell Sci. 1999;112 (Pt 7)1023-33.

[5] **Maw MA, Corbeil D, Koch J, Hellwig A, Wilson-Wheeler JC, Bridges RJ, et al.** A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. Hum Mol Genet. 2000;9(1):27-34.

[6] **Fargeas CA, Florek M, Huttner WB, Corbeil D.** Characterization of prominin-2, a new member of the prominin family of pentaspan membrane glycoproteins. J Biol Chem. 2003;278(10):8586-96.

[7] **Roper K, Corbeil D, Huttner WB.** Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. Nat Cell Biol. 2000;2(9):582-92.

[8] **Florek M, Bauer N, Janich P, Wilsch-Braeuninger M, Fargeas CA, Marzesco AM, et al.** Prominin-2 is a cholesterol-binding protein associated with apical and basolateral plasmalemmal protrusions in polarized epithelial cells and released into urine. Cell Tissue Res. 2007;328(1):31-47.

[9] **Barreda DR, Hanington PC, Walsh CK, Wong P, Belosevic M.** Differentially expressed genes that encode potential markers of goldfish macrophage development in vitro. Dev Comp Immunol. 2004;28(7-8):727-46.

[10] **Walsh JG, Barreda DR, Belosevic M.** Cloning and expression analysis of goldfish (*Carassius auratus* L.) prominin. Fish Shellfish Immunol. 2007;22(4):308-17.

[11] **Fixe P, Praloran V.** Macrophage colony-stimulating-factor (M-CSF or CSF-1) and its receptor: structure-function relationships. Eur Cytokine Netw. 1997;8(2):125-36.

[12] **Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y, et al.** Biology and action of colony--stimulating factor-1. Mol Reprod Dev. 1997;46(1):4-10.

[13] **Guilbert LJ, Stanley ER.** Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 1980;85(1):153-9.

[14] **Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER.** Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell. 1982;28(1):71-81.

[15] **Hamilton JA.** CSF-1 signal transduction. J Leukoc Biol. 1997;62(2):145-55.

[16] **Pixley FJ, Stanley ER.** CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol. 2004;14(11):628-38.

[17] **Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, et al.** Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. Science. 2008;320(5877):807-11.

[18] **Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al.** Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. J Leukoc Biol. 2010;88(3):495-505.

[19] **Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, et al.** IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. Cell Death Differ. 2010;17(12):1917-27.

[20] **Barreda DR, Hanington PC, Belosevic M.** Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol. 2004;28(5):509-54.

[21] **How GF, Venkatesh B, Brenner S.** Conserved linkage between the puffer fish (*Fugu rubripes*) and human genes for platelet-derived growth factor receptor and macrophage colony-stimulating factor receptor. Genome Res. 1996;6(12):1185-91.

[22] **Williams H, Brenner S, Venkatesh B.** Identification and analysis of additional copies of the platelet-derived growth factor receptor and colony stimulating factor 1 receptor genes in fugu. Gene. 2002;295(2):255-64.

[23] **Herbomel P, Thisse B, Thisse C.** Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001;238(2):274-88.

[24] **Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, et al.** Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. Mol Immunol. 2005;42(1):1-8.

[25] **Roca FJ, Sepulcre MA, Lopez-Castejon G, Meseguer J, Mulero V.** The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. Mol Immunol. 2006;43(9):1418-23.

[26] **Barreda DR, Hanington PC, Stafford JL, Belosevic M.** A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[27] **Mulero I, Pilar Sepulcre M, Roca FJ, Meseguer J, Garcia-Ayala A, Mulero V.** Characterization of macrophages from the bony fish gilthead

seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev Comp Immunol. 2008;32(10):1151-9.

[28] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[29] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[30] **Barreda DR, Neumann NF, Belosevic M.** Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Dev Comp Immunol. 2000;24(4):395-406.

[31] **Hanington PC, Wang T, Secombes CJ, Belosevic M.** Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[32] **Hanington PC, Hitchen SJ, Beamish LA, Belosevic M.** Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. Fish Shellfish Immunol. 2009;26(1):1-9.

[33] **Katzenback BA, Belosevic M.** Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney. Dev Comp Immunol. 2009;33(4):601-11.

[34] **Katzenback BA, Karpman M, Belosevic M.** Distribution and expression analysis of transcription factors in tissues and progenitor cell populations of the goldfish (*Carassius auratus* L.) in response to growth factors and pathogens. Mol Immunol. 2011;48(9-10):1224-35.

[35] **Naito M, Umeda S, Yamamoto T, Moriyama H, Umezu H, Hasegawa G, et al.** Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. J Leukoc Biol. 1996;59(2):133-8.

[36] **Naito M, Hasegawa G, Takahashi K.** Development, differentiation, and maturation of Kupffer cells. Microsc Res Tech. 1997;39(4):350-64.

[37] **Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al.** Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330(6005):841-5.

[38] **Houalla T, Levine RL.** The isolation and culture of microglia-like cells from the goldfish brain. J Neurosci Methods. 2003;131(1-2):121-31.

[39] **Lichanska AM, Browne CM, Henkel GW, Murphy KM, Ostrowski MC, McKercher SR, et al.** Differentiation of the mononuclear phagocyte system during mouse embryogenesis: the role of transcription factor PU.1. Blood. 1999;94(1):127-38.

[40] **Tagoh H, Himes R, Clarke D, Leenen PJ, Riggs AD, Hume D, et al.** Transcription factor complex formation and chromatin fine structure alterations at the murine c-fms (CSF-1 receptor) locus during maturation of myeloid precursor cells. Genes Dev. 2002;16(13):1721-37.

[41] **Sasmono RT, Ehrnsperger A, Cronau SL, Ravasi T, Kandane R, Hickey MJ, et al.** Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many other macrophage-specific transcripts and can transdifferentiate into macrophages *in vitro* in response to CSF-1. J Leukoc Biol. 2007;82(1):111-23.

[42] **Mathias JR, Dodd ME, Walters KB, Yoo SK, Ranheim EA, Huttenlocher A.** Characterization of zebrafish larval inflammatory macrophages. Dev Comp Immunol. 2009;33(11):1212-7.

[43] **Parichy DM, Ransom DG, Paw B, Zon LI, Johnson SL.** An orthologue of the kit-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, Danio rerio. Development. 2000;127(14):3031-44.

[44] **Barreda DR, Belosevic M.** Characterisation of growth enhancing factor production in different phases of *in vitro* fish macrophage development. Fish Shellfish Immunol. 2001;11(2):169-85.

[45] **Romano N, Picchietti S, Taverne-Thiele JJ, Taverne N, Abelli L, Mastrolia L, et al.** Distribution of macrophages during fish development: an immunohistochemical study in carp (*Cyprinus carpio*, L.). Anat Embryol (Berl). 1998;198(1):31-41.

[46] **Kollner B, Blohm U, Kotterba G, Fischer U.** A monoclonal antibody recognising a surface marker on rainbow trout (*Oncorhynchus mykiss*) monocytes. Fish Shellfish Immunol. 2001;11(2):127-42.

Chapter 6: Distribution and expression analysis of transcription factors in tissues, macrophage cell populations, and progenitor cell populations in response to myeloid growth factors and pathogens[3]

6.1 Introduction

The commitment and differentiation of a hematopoietic stem cell (HSC) and progenitor cell (HPC) into a functional mature cell is a tightly regulated process directed by the modulation of transcription factors that act to regulate gene expression [1-4]. Cytokines present in the hematopoietic cell niche influence the process of HSCs/HPCs expansion, survival and differentiation [5-7]. Cytokines that are involved in the myeloid developmental pathway include KIT ligand, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-stimulating factor-1 (CSF-1) and granulocyte colony-stimulating factor (GCSF). Kit ligand, also known as stem cell factor, is important for HSC self renewal [reviewed in [8]], mast cell development, and synergizes with other growth factors for the development of a variety of cell types [reviewed in [9]]. Other cytokines such as IL-3 and GM-CSF are important for the maintenance and development of myeloid precursors, while the CSF-1 and GCSF are required for the differentiation of precursors into monocytes/macrophages and granulocytes, respectively [10].

[3] A version of this chapter has been published: Katzenback *et al.*, 2011. *Molecular Immunology*, 48: 1224-1235.

The process of myelopoiesis responds to meet homeostatic cell turnover requirements, or to “emergency” situations instigated by an injury or infection to produce increased cell numbers of a particular lineage to meet the demands of the insult [11-13]. Often the production of one cell lineage is at the expense of another lineage; CSF-1 promotes monopoiesis at the expense of granulopoiesis, while GCSF creates the reverse situation [5, 14].

Ultimately, the action of myelopoietic growth factors is mediated through the expression of transcription factors. One example is CSF-1, which has been shown to induce *EGR1* in mouse bone marrow derived progenitors, an important transcriptional regulator in the development and differentiation of monocytes/macrophages [15, 16]. However, EGR1 antagonizes the transcription factor GFI-1, which is important for neutrophil differentiation [17, 18]. Many complex transcriptional networks involved in hematopoiesis have been elucidated and can be used to identify the particular stage of a cell within a lineage; one example is B- cell development [19].

In comparison, relatively little is known about teleost myelopoiesis. In part, progress is hampered by the lack of available reagents such as recombinant cytokines and antibodies. Alternatively, transcription factors, which appear to be conserved between higher and lower vertebrates, can be used to study developmental pathways in lower vertebrates. The expression of certain transcription factors has been used to identify cell types for studying hematopoiesis in the zebrafish model system [20-23].

In this chapter, I cloned and sequenced a number of goldfish transcription factors based on their involvement in the maintenance of HSCs/HPCs and in the differentiation of a HPC along a myeloid, erythroid, or lymphoid pathway. The

transcription factors RUNX1, c-Myb, and GATA2 are involved in the activation of myeloid genes and in the proliferation and maintenance of multipotent progenitor cells [24, 25]. The antagonism between PU.1 and GATA 1 regulates the myeloid/erythroid pathway and they were chosen as key transcription factors involved in this lineage fate decision [26]. EGR1, c-JUN, and MAFB were selected based on their involvement in monocyte/macrophage differentiation [15, 27-29], while CEBPα was chosen for its essential role in granulocyte development [30]. GATA1 and LMO2 were chosen for their ability to direct erythroid fate decisions and positively regulate erythroid differentiation [26, 31], and lastly, PAX5 and GATA3 are involved in B-cell [32] and T-cell [25] development, respectively.

Our laboratory has previously developed a unique *in vitro* fish primary kidney macrophage (PKM) culture system as a model system for monopoiesis that possesses all three populations of macrophage development: progenitor cells (R1s), monocytes (R3s), and macrophages (R2s) [33, 34]. The progenitor cells isolated from the kidney, the major hematopoietic organ in teleosts, are responsible for the generation of monocytes and macrophages. These cells are capable of proliferation and differentiation due to the production of endogenous growth factors that regulate these processes [reviewed in [35]]. I am particularly interested in the composition of the progenitor cell population, how these cells commit to a monocyte/macrophage lineage in the presence of a complex mixture of endogenous growth factors or defined cytokines, and lastly, how this progenitor cell population changes in response to pathogens *in vivo*. Therefore, the focus of this chapter was to examine these questions in relation to the goldfish kidney progenitor cell population using the panel of transcription factors I

identified as a means of understanding teleost myeloid progenitor cell development.

In this chapter, I present data that demonstrate the utility of transcription factors as potential markers of cell population and show that the differential regulation of transcription factors can be used to examine lineage fate decisions of progenitor cells in response to growth factors or pathogens. These novel data represent the first of their kind in any teleost system.

6.2 Results

6.2.1 Comparison of mRNA levels of hematopoietic transcription factors in goldfish kidney and spleen.

The mRNA levels of the transcription factors *runx1*, *cmyb*, *gata2*, *pu.1*, *mafb*, *cjun*, *egr1*, *cebp*α, *gata1*, *lmo2*, *gata3* and *pax5* in goldfish kidney and spleen tissues from four animals were analyzed by quantitative PCR. Transcription factors were grouped into four categories based on their major involvement in lineage decisions. The first group of markers of early progenitor cells consisted of the transcriptional regulators *runx1*, *cmyb*, and *gata2*. Expressions of these transcription factors were observed in the kidney, however, only *runx1* and *gata2* mRNAs were detected in the spleen, while *cmyb* mRNA was not detected (Fig. 6.1A).

The second group of transcription factors were involved in the macrophage/granulocyte cell lineages. The transcriptional regulators comprising this group include *pu.1*, *mafb*, *cjun*, *egr1*, and *cebp*α. The *cebp*α mRNA level was lower ($P < 0.08$), while the *cjun* mRNA level was higher ($P < 0.08$) in the

spleen compared to that of the mRNA levels in the kidney (Fig. 6.1B). However, mRNA levels of *pu.1*, *mafb,* and *egr1* did not significantly differ between kidney and spleen tissues (Fig. 6.1B).

The third group of transcription factors was selected based on their primary involvement in erythroid development-*gata1* and *lmo2*. The *gata1* mRNA levels were significantly higher in the spleen compared to mRNA levels in the kidney, while there was no difference in *lmo2* mRNA levels between tissues (Fig. 6.1C).

The last group of transcription factors were grouped together based on their involvement in lymphopoiesis; *gata3* as a marker of T-cell development and *pax5* as a master regulator of B-cell development. Both *gata3* and *pax5* mRNA levels were significantly higher in spleen compared to that of the kidney, $P < 0.05$ and $P < 0.08$, respectively (Fig. 6.1D).

6.2.2 Comparison of mRNA levels of hematopoietic transcription factors in goldfish cell populations.

I next examined the expression of different transcription factors in sorted R1 cells, peripheral blood leukocytes (PBLs), and splenocytes. Cell populations were isolated from three to four animals per each cell population. R1 cells from the kidney, the major hematopoietic organ of fish, consist of mainly progenitor cells. The *runx1* mRNA levels were significantly lower in splenocytes compared to that of R1 cells, whereas the relative mRNA levels of *runx1* in PBLs did not significantly differ from that in of R1 cells (Fig. 6.2A). Additionally, *cmyb* mRNA levels were significantly lower in PBLs and non-detectable in splenocytes

compared to that of R1 cells (Fig. 6.2A). However, *gata2* mRNA levels were significantly higher in both PBLs and splenocytes compared to those in the R1 cells (Fig. 6.2A). A similar trend was observed for the other transcription factors involved in the myeloid (Fig. 6.2B), erythroid (Fig. 6.2C), and lymphoid (Fig. 6.2D) pathways in which the relative expressions of transcription factors were significantly higher in PBLs and splenocytes compared to the sorted R1 cells.

6.2.3 Examination of the mRNA levels of hematopoietic transcription factors in R1 progenitor cells in comparison to whole kidney tissue

To elucidate the cell types isolated from the kidney tissue that compose the R1 progenitor cell population, I directly compared the R1 cell population to that of the whole kidney tissue. Kidney tissue from four animals, or sorted R1 progenitor cells from freshly isolated kidney cells from four fish were used in this experiment. The transcription factors *gata2*, *cjun* and *egr1* had significantly higher mRNA levels in the R1 population of cells compared to those in the kidney, while mRNA levels of *cebp*α and *pu.1* were significantly lower in R1 cells compared to the kidney (Fig. 6.3A and B). The mRNA levels of *pax5* and *gata3*, transcription factors involved in the lymphoid lineage, were also significantly higher in R1 cells compared to those in the kidney tissue (Fig. 6.3D). The mRNA levels of *lmo2* were significantly lower in R1 cells compared to the kidney tissue, and a significant up-regulation of *gata1* mRNA levels were observed in R1 progenitor cells compared to the kidney tissue, $P < 0.08$ (Fig. 6.3C). As expected, the R1 progenitor cell population expressed markers for HSCs/HPCs and lymphocytes.

6.2.4 Hematopoietic transcription factor mRNA levels in progenitors, monocytes, and macrophages

The development of progenitor cells along the macrophage lineage is directed by transcription factor expression. To examine the transcription factors expressed at different stages of macrophage development, I sorted progenitor cells, monocytes and macrophages from PKM cultures and determined the relative mRNA levels of the panel of transcription factors. For these experiments, R1 progenitor cells were sorted from day 0, or freshly isolated kidney cells, monocytes were sorted from day 2-4 primary kidney macrophage cultures, and macrophages were sorted from day 6-8 primary kidney macrophage cultures. For each cell sub-population, cells were obtained from individual PKM cultures from four fish. Quantitative PCR was used to determine the mRNA levels for each transcription factor.

The mRNA levels of *runx1* were significantly increased in monocytes, but not macrophages, compared to progenitor cells, whereas the levels of *gata2* mRNA were significantly reduced in macrophages compared to progenitor cells (Fig. 6.4A). No significant differences were observed in *cmyb* mRNA levels among progenitors, monocytes or macrophages (Fig. 6.4A). The mRNA levels of *pu.1* were significantly upregulated in monocytes by approximately 8 fold compared to progenitor cells, while macrophages did not have significantly higher mRNA levels of *pu.1* compared to that of progenitor cells (Fig. 6.4B). When I examined the mRNA levels of *mafb*, *cjun*, and *egr1* a similar trend was seen among them; macrophages had significantly lower mRNA levels of *mafb*, *cjun*,

and *egr1* in comparison to both progenitor cells and monocytes (Fig. 6.4B). No significant changes in *cebp*α mRNA levels were observed (Fig. 6.4B), or in the mRNA levels of the transcription factors involved in erythropoiesis (Fig. 6.4C). The mRNA levels of the transcription factors involved in lymphopoiesis, *gata3* and *pax5*, were both significantly lower in monocytes and macrophages compared to the mRNA levels in progenitor cells (Fig. 6.4D).

6.2.5 Comparison of mRNA levels of hematopoietic transcription factors in R1 progenitor cells isolated from PKM cultures

The kidney is the major hematopoietic organ of fish and previous studies have shown that following cultivation of kidney R1 progenitor cells for 4 to 10 days in the presence of cell-conditioned medium (CCM) or rgCSF-1 results in the generation of monocytes and mature macrophages [33, 34, 36-39]. Therefore, I wanted to examine how the R1 cell population changes over time in culture as monocytes and macrophage are generated. R1 cells were sorted at day 0, day 2, or day 6 of culture from three individual PKM cultures at each time point, and the expression of the different transcription factors normalized to that of the day 0 R1 cell population.

The mRNA levels of the transcriptional marker of early progenitor cells, *gata2,* were significantly higher in the day 2 R1 cells compared to that at day 0, and were significantly lower in day 6 R1 cells compared to that in day 0 cells (Fig. 6.5A). No significant differences were observed for *cmyb* or *runx1* mRNA levels at any time point (Fig. 6.5A). The expression of *mafb* was significantly higher in day 2 R1 cells compared to day 0 cells by approximately 6-fold (Fig. 6.5B), and

was significantly lower by day 6 of cultivation compared to day 0 R1 progenitor cells. While mRNA levels of *cjun* did not significantly differ in day 0 and day 2 R1 progenitor cells, the mRNA levels of this transcription factor were significantly lower in day 6 R1 progenitor cells compared to day 0 R1 cells (Fig. 6.5B). The mRNA levels of erythroid and lymphoid transcriptional regulators exhibited a pattern in which mRNA levels were significantly increased by day 2 of cultivation followed by a significant decrease at day 6 of cultivation compared to day 0 progenitor cells (Fig. 6.5C and D).

6.2.6 Effects of recombinant goldfish KITLA and CSF-1 on transcription factor expressions in day 2 progenitor cells

Recombinant goldfish CSF-1 has previously been functionally characterized and has been shown to be involved in the proliferation and differentiation of macrophages and their progenitors [39, 40], whereas rgKITLA has been shown to be involved in the survival of goldfish progenitor cells [41] (described in chapter 4). I then examined whether these growth factors were involved in the regulation of transcription factors associated with these functional processes. Therefore, to investigate the effects of recombinant goldfish growth factors on transcription factor expression in progenitor cells *in vitro*, I treated day 2 progenitor cells with rgKITLA, rgCSF-1, or rgKITLA and rgCSF-1 in combination. Not surprisingly, I observed that many of the myeloid and early progenitor transcription factors were modulated by these growth factors. Treatment of day 2 progenitors with rgCSF-1 for 6 hrs induced a significant up-regulation in the mRNA levels of the transcriptional regulators *cjun* and *egr1* (Fig.

6.6B). The expression of transcription factors involved in early progenitor cells, erythropoiesis and lymphopoiesis were not significantly different from medium treated controls (Fig. 6.6A, C and D).

Upon treatment of the day 2 progenitor cells with rgKITLA, I observed a significant up-regulation of the mRNA levels of *mafb*, *egr1*, and *cebpα* (Fig. 6.6B). Similar to treatment with rgCSF-1, there were no significant differences between non-treated and rgKITLA-treated early progenitors when the mRNA levels of transcription factors from the early progenitor cells, erythroid and lymphoid pathways were measured (Fig. 6.6A, C and D). Previous studies examining the function of kit ligand, also known as stem cell factor (SCF) in mammalian literature, found it to be synergistic with other cytokines. Due to these observations, I also treated sorted day 2 progenitors with a combination of rgKITLA and rgCSF-1. The mRNA levels of the myeloid transcription factors *egr1* and *cebpα* (Fig. 6.6B) were significantly up regulated compared to time matched controls.

6.2.7 Transcription factor mRNA levels in day 1, day 2, and day 3 sorted progenitors treated with recombinant goldfish growth factors

Based on the increased mRNA levels of *gata2* and *mafb* of day 2 progenitors compared to that of day 0 R1 progenitor cells, I next wanted to characterize the population of progenitor cells present at different time points in culture. Progenitor cells were sorted from day 1, day 2 and day 3 PKM cultures for use in growth factor treatment experiments. At each time point, three to four PKM cultures were used. I chose these time points based on previous changes in

constitutive transcription factor mRNA levels in sorted progenitor cells from different days of PKM culture [42]. Based on this previous data, I believed the population of progenitor cells to change over time in culture with progenitor cells becoming committed to the macrophage lineage by day 2 of cultivation [42], as described in section 6.2.5. In addition, the immunofluorescence results using the rabbit anti-sCSF-1R IgG antibody binding to progenitor cells showed a decrease in the fluorescence intensity of progenitors positive for CSF-1R and supported my previous observations of a dynamic progenitor cell population (the subject of chapter 5). To determine whether the changes observed in transcription factor mRNA levels and changes in rabbit anti-sCSF-1R IgG binding corresponded to functional changes in the progenitor cells, I treated sorted progenitor cells from different days of cultivation with recombinant growth factors. Sorted progenitors were treated with medium alone, 100 ng/mL rgCSF-1, 100 ng/mL rgKITLA or a combination of 100 ng/mL rgCSF-1 and rgKITLA. Cells were exposed to the recombinant growth factors for 0.5 hrs, 3 hrs, or 6 hrs prior to determination of mRNA levels by quantitative PCR.

I chose to focus on the response of early progenitor and myeloid transcription factors to recombinant growth factors. The first experiment examined the response of transcription factor mRNA levels in progenitor cells treated with rgCSF-1. The mRNA levels of *cebp*α and *cjun* were down regulated in day 1 progenitor cells treated with rgCSF-1, whereas *egr1* mRNA levels were significantly upregulated in rgCSF-1 treated day 1 progenitor cells compared to that of the medium control at 0.5 hrs post treatment (Fig. 6.7A). However, by 3 and 6 hours post treatment with rgCSF-1, the mRNA levels of *cebp*α, *cjun*, and

egr1 were not significantly different from that of the medium controls (Fig. 6.7A). No significant changes were observed in the mRNA levels of *runx1*, *gata2*, *mafb*, or *gata2* in day 1 progenitor cells treated with rgCSF-1 at any time point measured (not shown). When day 2 progenitor cells were treated with rgCSF-1, a significant ($P < 0.08$) increase was observed in *cebpa* mRNA levels at 3 hrs post treatment compared to that of the medium control, but retuned to basal levels by 6 hrs (Fig. 6.7B). Six hours post treatment of day 2 progenitor cells with rgCSF-1, a significant increase was observed in the mRNA levels of *cjun* and *egr1* compared to those of the medium control (Fig. 6.7B). No significant changes were observed in the mRNA levels of *runx1*, *mafb* or *gata2* in the day 2 progenitor cells treated with rgCSF-1 at any time point (not shown). The mRNA levels of both *runx1* and *cebp*α showed a general trend of down regulation at 0.5 hrs and 3 hrs post rgCSF-1 treatment and were significantly down-regulated in day 3 progenitor cells treated with rgCSF-1 at 6 hrs (Fig. 6.7C). The mRNA levels of *mafb* were increased in day 3 progenitor cells treated with rgCSF-1 by 6 hrs, $P < 0.08$(Fig. 6.7C). No significant changes were observed in the mRNA levels of *cjun*, *egr1*, *gata2*, or *pu.1* in day 3 progenitor cells treated with rgCSF-1 at any time point (not shown).

I next assessed the regulation of transcription factor mRNA levels in progenitor cells treated with rgKITLA. In day 1 progenitor cells, there was a significant decrease in *runx1*, *cebp*α and *cjun* mRNA levels by 6 hrs post rgKITLA treatment compared to the medium control (Fig. 6.8A). However, in day 2 progenitor cells, there was a decrease in the mRNA levels of *cebp*α by 3 hrs ($P < 0.08$), followed by an increase in the mRNA levels of *cebp*α, *egr1*, and *mafb* by

6 hours post treatment with rgKITLA compared to the mRNA levels of the medium controls (Fig. 6.8B). A generalized decrease in the mRNA levels of *cebpα*, *cjun*, and *egr1* was observed in day 3 progenitors treated with rgKITLA at all time points (Fig. 6.8C). The mRNA levels of *cebpα*, *cjun* and *egr1* were decreased at 3 hrs, while only *cebpα* mRNA levels were significantly decreased by 6 hrs from day 3 progenitor cells treated with rgKITLA compared to the time matched medium controls (Fig. 6.8C).

Lastly, I examined the treatment of progenitor cells with a combination of rgCSF-1 and rgKITLA. Upon treating day 1 progenitor cells with the combination of growth factors, there was an initial decrease in the mRNA levels of *runx1* and *cebpα* at 0.5 hrs compared to that of the time-matched medium controls (Fig. 6.9A). The mRNA levels of *runx1* and *cebpα* in sorted day 1 progenitor cells were not significantly different from their respective time-matched medium controls at 3 and 6 hrs post treatment with rgCSF-1 and rgKITLA (Fig. 6.9A). Treatment of day 2 sorted progenitor cells with the combination of rgCSF-1 and rgKITLA showed no significant change in transcription factor mRNA levels at 0.5 hrs and 3 hrs (Fig. 6.9B). However, at 6 hrs, the mRNA levels of *cebpα* and *egr1* were significantly increased in day 2 progenitor cells compared to their respective time matched medium controls (Fig. 6.9B). Day 3 progenitor cells treated with rgCSF-1 and rgKITLA did not exhibit any significant changes in myeloid transcription factor expression until 6 hrs post treatment. At 6 hrs post treatment, the mRNA levels of *runx1* and *cebpα* were significantly decreased, $P < 0.08$ and $P < 0.05$, respectively, compared to the time-matched medium controls (Fig. 6.9C).

Taken together, these data show a differential response of transcription factor mRNA levels in progenitor cells sorted from different days of cultivation to growth factors. The differential regulation of transcription factor mRNA levels in day 1, 2, or 3 sorted progenitor cells suggests that endogenous growth factors produced by these cells within culture act to influence progenitor cell commitment and responsiveness to growth factors.

6.2.8 Expressions of hematopoietic transcription factors in R1 progenitor cells isolated from heat-killed *A. salmonicida* A449 challenged goldfish.

Due to the conserved nature of the transcription factors and the pathways they are involved in, I decided to examine the expressions of transcription factors *in vivo* in order to assess the early effects of natural fish pathogens on developing progenitor cells. In the first set of experiments, I challenged four goldfish with 1 x 10^9 heat-killed *A. salmonicida* or sham-injected PBS controls, isolated and sorted R1 cells from the kidney 72 hours post challenge and analyzed different transcription factor mRNA levels by quantitative PCR. Following challenge with *A. salmonicida*, changes in the transcription factor mRNA levels were observed in the early progenitor and myeloid groups, while no significant differences were observed within the erythroid (Fig. 6.10C) or lymphoid (Fig. 6.10D) groups of transcription factors. Within the early progenitor group, a significant decrease in *runx1* mRNA levels ($P < 0.05$) and in *cmyb* mRNA levels ($P < 0.08$) were observed in R1 cells from heat-killed *A. salmonicida* challenged fish compared to PBS-injected control fish (Fig. 6.10A). In the myeloid group of transcription factors, a significant increase in *pu.1* ($P <$

0.05) and in *cjun* mRNA levels ($P < 0.08$) were observed in R1 cells isolated from the heat-killed *A. salmonicida* exposed goldfish compared to the PBS-injected controls (Fig. 6.10B). However, the level of *egr1* mRNA was significantly lower in the R1 cells from the *A. salmonicida* challenged group (Fig. 6.10B).

6.2.9 Regulation of transcription factors in *T. carassii* infected goldfish

The second pathogen I chose to examine was *T. carassii*, an extracellular protozoan parasite of cyprinid fish. In the first set of experiments, four fish were injected with PBS (sham-injected control) or 1 x 10^8 trypanosomes and R1 cells from the kidney were isolated and sorted 72 hours post infection. The mRNA levels of the transcription factors from the early progenitor group *runx1*, *cmyb* and *gata2* were significantly lower in the sorted R1s from trypanosome infected fish compared to the sorted R1s from the PBS-injected control fish (Fig. 6.11A). A similar trend was observed for the expression of transcription factors in the myeloid, erythroid, and lymphoid groups. The mRNA levels of the transcription factors *mafb*, *egr1* and *cebp*α were all significantly lower in R1s from fish infected with *T. carassii* (Fig. 6.11B), while the mRNA levels of *pu.1* and *cjun* did not change. Furthermore, the mRNA levels of *gata1* (Fig. 6.11C), *gata3* and *pax5* (Fig. 6.11D) were significantly lower in R1 cells from *T. carassii* infected fish compared to PBS-injected controls.

In the second set of experiments, four fish were injected with PBS (sham injected controls) or infected with 6.25 x10^6 trypanosomes and kidney and spleen tissues isolated at 7 days post infection. A lower dose of parasites was chosen to

infect fish for this experiment compared to the first experiment, as high numbers of parasites within the host blood have been shown to cause host mortality. After exposure to 6.25 x 10^6 trypanosomes, the parasites multiply within the host and reach peak parasitemia two to three weeks post infection, followed by host recovery by 8 weeks post infection. Therefore at 7dpi, fish infected with 6.25 x 10^6 will have approximately the same parasite burden as those fish infected with 1 x 10^8 trypanosomes at 3 dpi.

In contrast to the first set of experiments, in which I observed a generalized decrease in transcription factor mRNA levels in R1 cells isolated from trypanosome infected fish (Fig. 6.11), the mRNA levels of the transcription factors in the kidney at 7dpi from *T. carassii* infected fish, except for *runx1* which was significantly lower in *T. carassii* infected fish, did not significantly differ from the PBS-injected controls (Fig. 6.12A-D). However, when I examined the transcription factor mRNA levels in the spleen, there was a significant decrease in the levels of *pu.1*, *mafb* and *cjun* (Fig. 6.13B) in *T. carassii* infected fish. No significant differences were observed in the expression of erythroid (Fig. 6.13C) or lymphoid (Fig. 6.13D) transcription factors. Although the relative mRNA levels of many of the transcription factors did not significantly differ between spleen tissue from PBS controls and *T. carassii* infected fish, there was a general trend of lower of the expression of all transcription factors in the spleen of trypanosome infected fish (Fig. 6.13).

6.3 Discussion

To increase our understanding of teleost HSCs/HPCs I chose to use transcription factor mRNA levels as a tool to examine how progenitor cell populations are influenced by growth factors and in response to pathogens. cMYB is a marker of definitive HSCs in developing zebrafish [43] and is required for the production of all blood cells, suggesting an evolutionarily conserved role for cMYB in hematopoiesis [44]. In my study, I observed enhanced *cmyb* mRNA levels in the kidney tissue and kidney R1 progenitor cells consistent with the hematopoietic role of the kidney in teleosts, and suggests that the R1 progenitor cell population most likely contains a population of HSCs. This is in agreement with previous reports of isolation of a side-population of cells believed to contain HSCs from adult ginbuna carp kidney [45-47]. Surprisingly, *cmyb* expression in PBLs was observed, albeit at very low levels, and may suggest the presence of a small fraction of circulating HSCs in the blood of teleosts. RUNX1 and GATA2 are also involved in early hematopoiesis. RUNX1 is well known for its role in the formation of HSCs [48] and is expressed during T-cell development, particularly in double-negative, double-positive, and $CD4^+$ and $CD8^+$ T-cells [reviewed in [49]]. The similar levels of *runx1* in goldfish kidney and spleen tissues may be due to the expression of this transcription factor in HSCs and T-cells, with the majority of HSCs being found in the kidney and a population of T-cells contained within the spleen. GATA2, while important for the survival and proliferation of multipotent progenitors, is broadly expressed amongst megakaryocytes and cells from the mast cell lineage [50]. The highly significant increase in *gata2* expression in PBLs and splenocytes compared to R1 progenitor cells may be due

to the presence of mast cells, since GATA2 has recently been shown to be important in the development of zebrafish mast cells [51, 52]. Similarly, increased mRNA levels of the transcription factors *cjun*, *egr1*, *gata1*, *gata3* and *pax5* in the spleen most likely reflect the expression of these transcription factors in mature cells within the spleen, splenocytes and PBLs populations such as monocytes/macrophages [53-55], erythrocytes, megakaryocytes, mast cells [56], T-cells [25] [57] and B-cells [58]. Of particular interest were the lower mRNA levels of *cebp*α in the spleen compared to those in the kidney. CEBPα is important for the production of granulocytes, and I have previously isolated and characterized a pool of neutrophils from the goldfish kidney [41]. These results are consisting with our findings of large numbers of neutrophils in the kidney, and further supports the teleost kidney as the site of granulopoiesis.

The decision to commit to the macrophage lineage and develop into mature macrophages is mediated by growth factors and transcription factors. Therefore, the surface expression of receptors and transcription factors can be utilized as markers of cell lineage [59]. To achieve a better understanding of the transcription factors that may be involved in the development of progenitor cells into monocytes and macrophages, I surveyed the relative mRNA levels of a panel of goldfish transcription factors. The significant increase in *runx1* and *pu.1* mRNA levels in monocytes compared to the mRNA levels in progenitor cells suggests that *runx1* and *pu.1* may play a vital role in the developing monocyte population. RUNX1, PU.1 and CEBPα binding sites have all been identified in the regulation of CSF-1R mRNA expression (*fms* gene) in mice [60, 61]. Thus, the increase in *runx1* and *pu.1* in goldfish monocytes may be due to the increasing

expression of CSF-1R that is observed during macrophage development [10]. In the case of *mafb*, *cjun*, and *egr1* mRNA levels, there were no significant differences between the mRNA levels of progenitor cells and monocytes, but a significant decrease in the mRNA levels of the transcriptions factors in macrophages. These data suggest that *mafb*, *cjun*, and *egr1* may be important during development, but once myeloid cells are terminally differentiated into mature macrophages, these transcription factors are no longer required. The down-regulation of Egr families and *RUNX1* and *RUNX3* were also down-regulated in monocyte to macrophage *in vitro* differentiation from healthy human donors [62]. However, *CEBP*α was up-regulated in macrophages compared to monocytes [62], which was not the case when I compared goldfish *cebp*α mRNA levels in monocytes and macrophages. In a study examining macrophage development in mice, they noted a similar increase in *CEBP*α mRNA early during macrophage development, with a slight decrease in mature macrophages, while the mRNA levels of *GATA1*, *GATA2*, *PU.1* and *RUNX1* were decreased in mature macrophages compared to progenitor cells [60]. Therefore, it appears the overall expression of transcription factors in progenitors, monocytes, and macrophages in goldfish are similar to that observed in higher vertebrates. The involvement of CEBPα in goldfish macrophage development may be an interesting avenue to pursue.

Due to the importance of transcription factors at multiple junctures of developing immune cells and the development of multiple cell lineages, I focused my efforts on the detailed examination of the expression of transcription factors in the R1 progenitor cell population during cultivation. R1 progenitor cells compared

to kidney tissue showed increased mRNA expressions of *gata2* and *egr1*, which have previously been identified as markers of HSCs in humans, mice and zebrafish [63]. The expression of *cjun*, *pax5* and *gata3* were also increase in sorted goldfish progenitor cells compared to that of kidney tissue. These data suggest that the cultivation system of PKM is enriching for a mixed population of the progenitor cells, most likely containing a small number of HSCs, myeloid progenitors, and quite possibly a small population of lymphoid progenitors or mature lymphocytes. Furthermore, upon examination of the R1 progenitor cell population over time in the PKM culture system, I believe the dynamic changes in the expression of many of the transcription factors involved in HSCs/HPCs, erythroid or lymphoid lineages may be due to the cell death that occurs by day 2 in our culture system (lag phase). MAFB is important in the self-renewal of progenitor cells [28, 29], and a decrease in MAFB in progenitors results in an up-regulation of *PU.1* and in increase in cell responsiveness to CSF-1[29] to permit monocyte/macrophage differentiation which requires MAFB expression [28, 29]. Similarly, GATA2 is important for the maintenance and proliferation of multipotent progenitor cells [25], as well as inhibition of monocyte/macrophage differentiation by suppressing PU.1 [64]. Increased expression of *mafb* and *gata2* mRNA levels in day 2 R1 progenitors suggests that progenitor cell commitment to the macrophage lineage may be suppressed at this time, and that this block to monocyte/macrophage differentiation is removed following day 2 of culture, coinciding with the end of lag phase and the beginning of the proliferative phase and macrophage outgrowth. Conversely, the up-regulation of *mafb* mRNA levels in the R1 population may be occurring in a sub-population of cells that is independent from the sub-populations of cells expressing *gata2* mRNA, which

suggests that commitment of progenitors to a macrophage lineage may occur at day 2 of cultivation.

Treatment of sorted day 2 progenitor cells with rgCSF-1 showed up-regulation of the myeloid transcription factor mRNA levels *cjun*, *egr1*, and *cebp*α, known to be involved in the differentiation of myeloid progenitors into monocytes/macrophages [24, 27, 65]. Possibly, the endogenous production of CSF-1 by alternative macrophages present early in PKM cultures [34, 38] may signal for the commitment, proliferation and differentiation of the day 2 R1 progenitor cells, thereby ending the lag phase and signaling the entrance into the proliferative phase of PKM cultures [36]. The mixture of endogenous growth factors produced by the PKM cells is complex. Thus far, studies in the Belosevic lab have identified and functionally characterized the following growth promoting and differentiation factors: CSF-1, LIF, granulin and KITLA [39, 66-68]. Since KIT ligand is known to act during early hematopoiesis, I assessed whether it affected the expression of the transcription factors in progenitor cells. The observed increase in *mafb* and *egr1* expressions support the functional role of rgKITLA as a promoter of progenitor cell survival [68], as EGR1 is known to influence the maintenance of HSCs quiescence and location within the bone marrow niche [69]. The up-regulation of *cebp*α mRNA in response to treatment with rgKITLA suggests that rgKITLA may be involved in fish mast cell development similar to the mammalian system [9]. The treatment of progenitor cells with the combination of growth factors maintained the up-regulation in the expression of the myeloid transcription factors *egr1* and *cebp*α and suggests that rgKITLA and rgCSF-1 may synergize to induce differentiation along the monocyte/macrophage lineage.

In addition, I decided to treat sorted progenitor cells from day 1, day 2, or day 3 of culture with the recombinant myeloid growth factors, rgCSF-1, rgKITLA or a combination. These experiments demonstrated the differential regulation of transcription factors by myeloid growth factors based on the day of progenitor cell isolation. In particular, the increase in mRNA levels of myeloid transcription factors (*cebp*α, *cjun*, and *egr1*) in day 2 sorted progenitor cells after 6 hours of treatment with rgCSF-1, rgKITLA, or a combination of the two growth factors, suggests the exposure of these progenitor cells to endogenous growth factors that may act to "prime" the progenitor cells to become responsive to rgCSF-1 or rgKITLA. Unlike differentiated cells, progenitor cells require multiple signals, in the form of growth factors, to induce a response, reviewed in [70]. Conversely, the differential response of sorted progenitor cells from different days of culture when treated with growth factors may reflect the changes in cell population composition within the progenitor cell gate. In addition, regardless of the day of progenitor cell isolation, Idid not observe a rapid (by 0.5 hr) response in *egr1* mRNA levels in goldfish progenitor cells treated with rgCSF-1. This is in contrast to experiments performed on isolated mouse bone marrow cells treated with mouse CSF-1, where CSF-1 induced a rapid and significant increase in *EGR1* mRNA levels in mouse bone marrow isolates [15]. I believe this may represent a differential regulation of CSF-1 signaling in fish macrophages in comparison to mouse macrophage precursors. Conversely, these data could signify the differences in the composition of the precursor pool isolated from the hematopoietic niche of fish or higher vertebrates such as mice.

I then examined how the HSC/HPC pool was regulated *in vivo* in response to pathogens. The regulation of the transcription factor mRNA levels in

R1 progenitors isolated from heat-killed *A. salmonicida* challenged fish suggests a shift towards early myeloid committed progenitors. However, down-regulation of *runx1* and *egr1* mRNA levels may suggest an arrest in commitment/progression of these progenitors to the monocyte/macrophage lineage, or possibly the mobilization of these cells from the hematopoietic organ to the periphery. Similar observations were reported for *Listeria monocytogenes* infection of mice, where there was a decrease of myeloid precursors from days 1-4 post infection. However, as the infection progressed, the numbers of myeloid progenitors recovered and there was an enhancement of monopoiesis at the expense of other cell lineages [71].

The progenitor cells from *T. carassii* infected fish showed different expression profiles of transcription factors. The generalized down-regulation of transcription factor expressions may be explained by the immunosuppressive environment that trypanosomes are known to induce, as well as the added demand on the hematopoietic system to replace dying cells. Similar observations have been reported for other pathogen/host systems. For example, immunosuppression in mice caused by *T. brucei* infection was related to significant (50%) reduction in the nucleated cells from the bone marrow following peak parasitemia, and the capacity of equal numbers of these cells to form spleen-colony-forming units was diminished by more than 50% [72]. A decrease in the number and colony-forming unit potential of myeloid and erythroid progenitors was also seen in cytomegalovirus infected BALB/c mice [73]. However, by 7 days post infection the only significant down-regulation was in *runx1* mRNA levels in the kidney tissue from *T. carassii* infected fish. This may suggest that after pathogenic insult the recovery and return to homeostasis in the

goldfish kidney is relatively rapid. In the spleen of infected fish, a general trend in down-regulation of transcription factor mRNA levels may suggest a lack of mature cells present in this tissue.

Despite the lack of available reagents to examine teleost progenitor cells, I have employed the conserved nature of transcription factors as a tool to gain a better understanding of this important population of cells. By determining the expression of different transcription factors under different conditions, I examined the dynamics of the sorted progenitor cell populations in our unique PKM cultivation system and how growth factors influence these cells. My results provide a basis for using the transcription factor expression as a marker for development and differentiation status of myeloid cells. Furthermore, while many studies have examined how mature immune cells respond to pathogens, comparative immunologists have yet to systematically examine how pathogens are able to modulate the progenitor cell pools in the kidney of the bony fish. My results indicate that there is significant modulation of HSCs/HPCs during “emergency” conditions in the teleost kidney. Whether this modulation is directly influenced by pathogen molecules or is a result of generalized increase in the hematopoietic demand remains to be elucidated.

To my knowledge, this is the first report examining the relative abundance of transcription factor mRNA levels in teleost mononuclear phagocyte lineage populations (progenitors, monocytes, macrophages), and the regulation of transcription factor mRNA levels in teleost progenitor cells treated with defined recombinant myeloid growth factors.

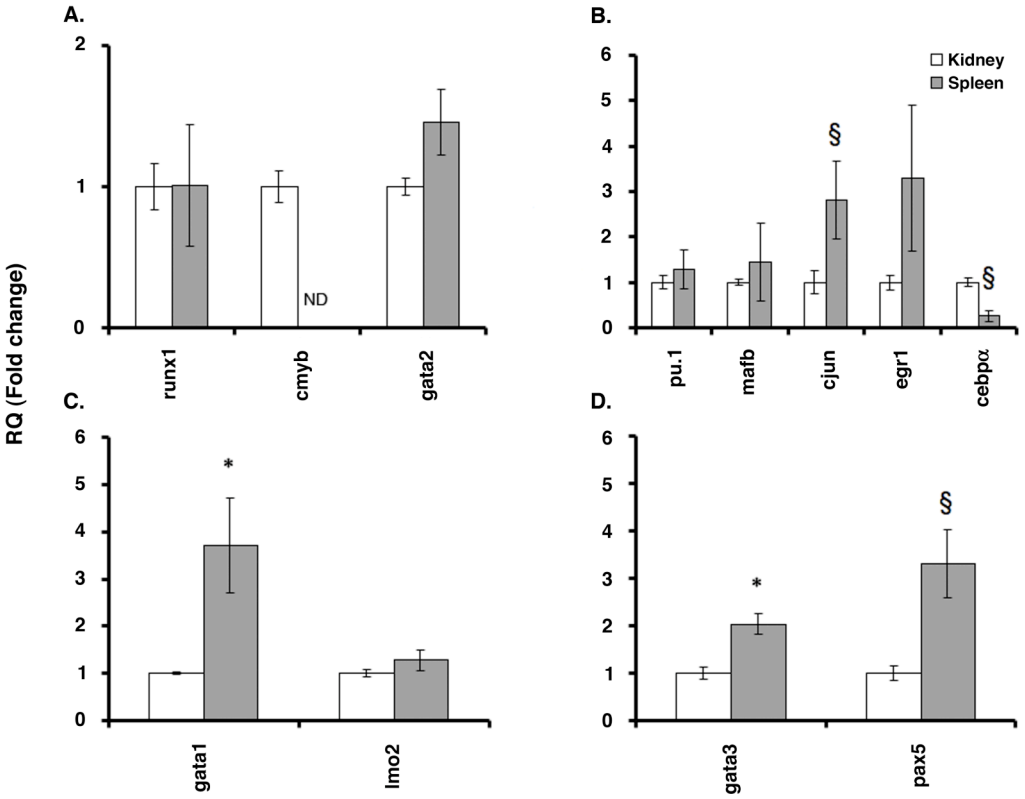


Figure 6.1: Comparison of hematopoietic transcription factor expressions between kidney and spleen of goldfish using quantitative PCR.
Quantitative PCR was used to assess the mRNA levels of the transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in kidney and spleen tissues. Data was normalized to the kidney and standard error is shown. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§). ND, not detected, ($n = 4$).

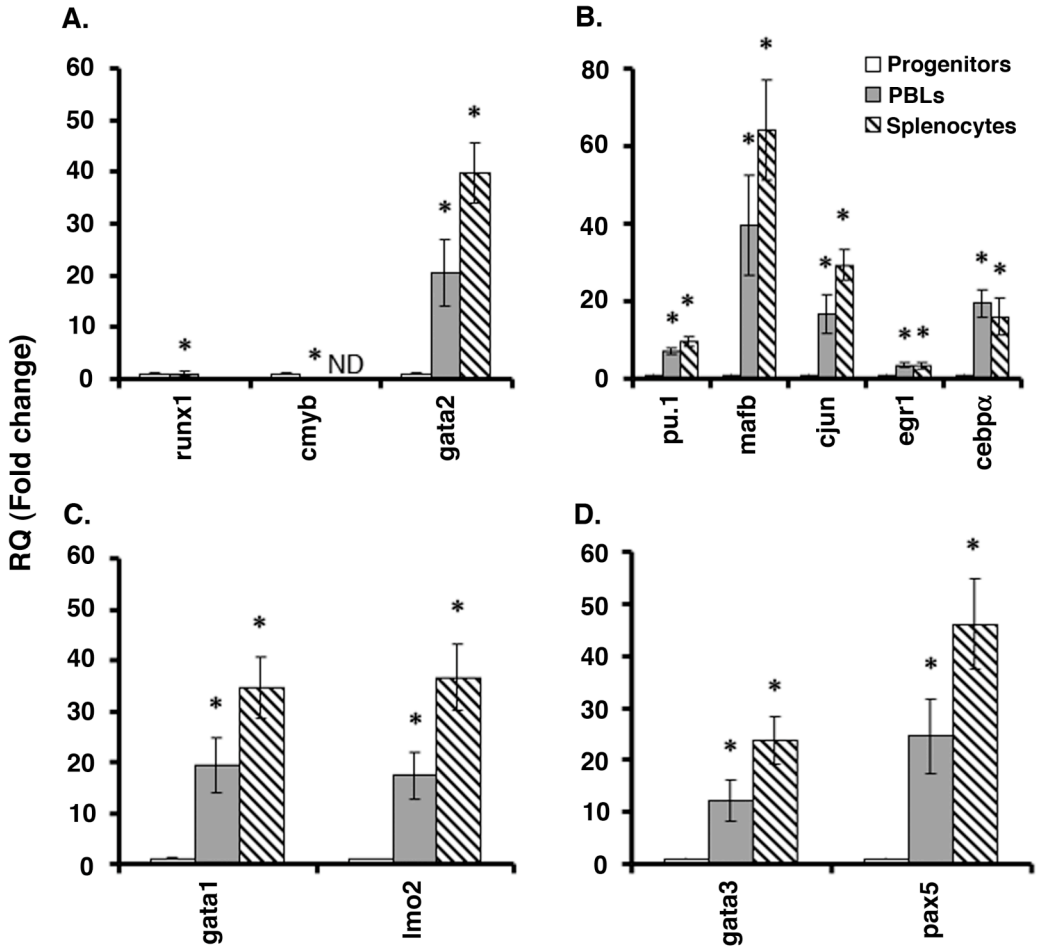


Figure 6.2: Comparison of hematopoietic transcription factor expressions in goldfish cell populations using quantitative PCR.
Quantitative PCR was used to asses the mRNA levels of transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) was assessed in R1 progenitor cells (R1s), peripheral blood leukocytes (PBLs), and splenocytes. Data was normalized to the R1 progenitor cells and standard error is shown. Significance is denoted by (*) compared to the reference sample ($P < 0.05$). ND, not detected, ($n = 4$).

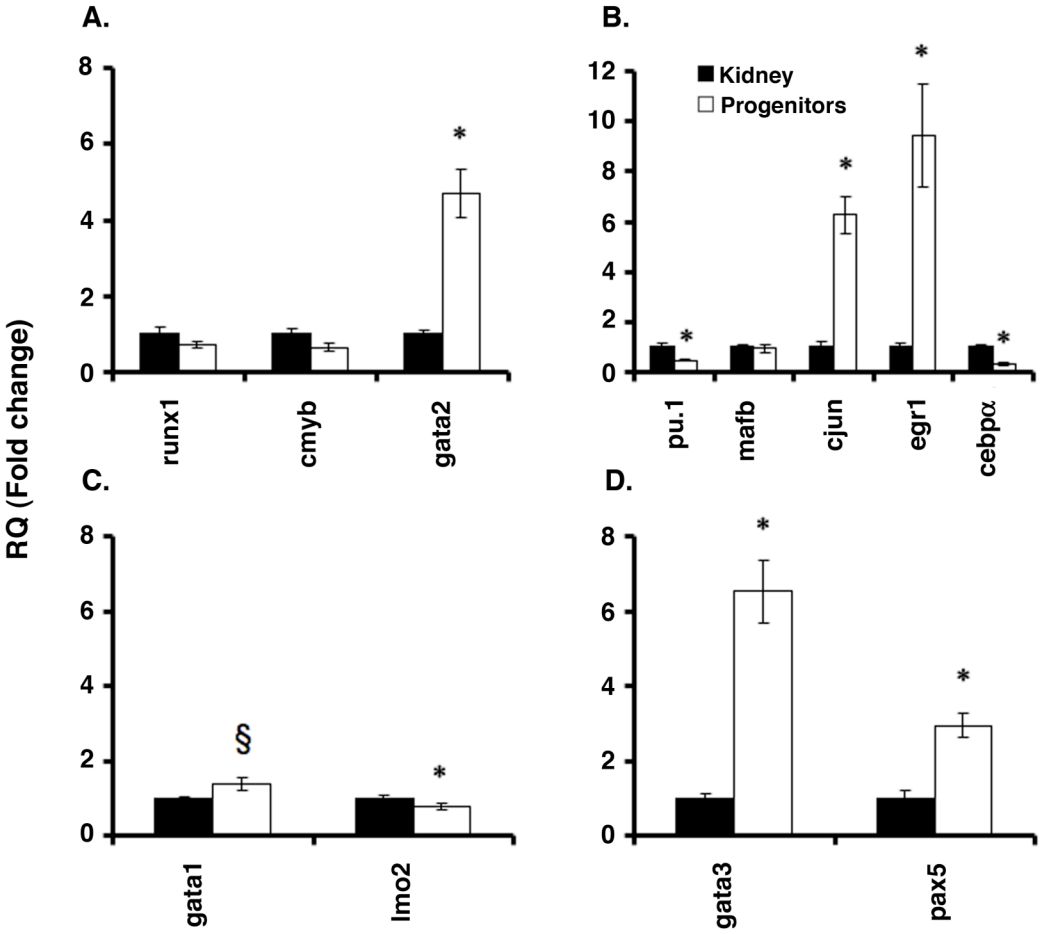


Figure 6.3: Comparison of hematopoietic transcription factor expressions in goldish whole kidney tissue and sorted R1 progenitor cells using quantitative PCR.

Kidney tissue isolated from four animals, or freshly isolated and sorted R1 progenitor cells from another four animals were used to determine transcription factor expressions using quantitative PCR. The mRNA levels of transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in R1 progenitor cells (R1s) and kidney tissue were assessed. Data were normalized to the kidney tissue and standard error is shown. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§). ND, not detected, ($n = 4$).

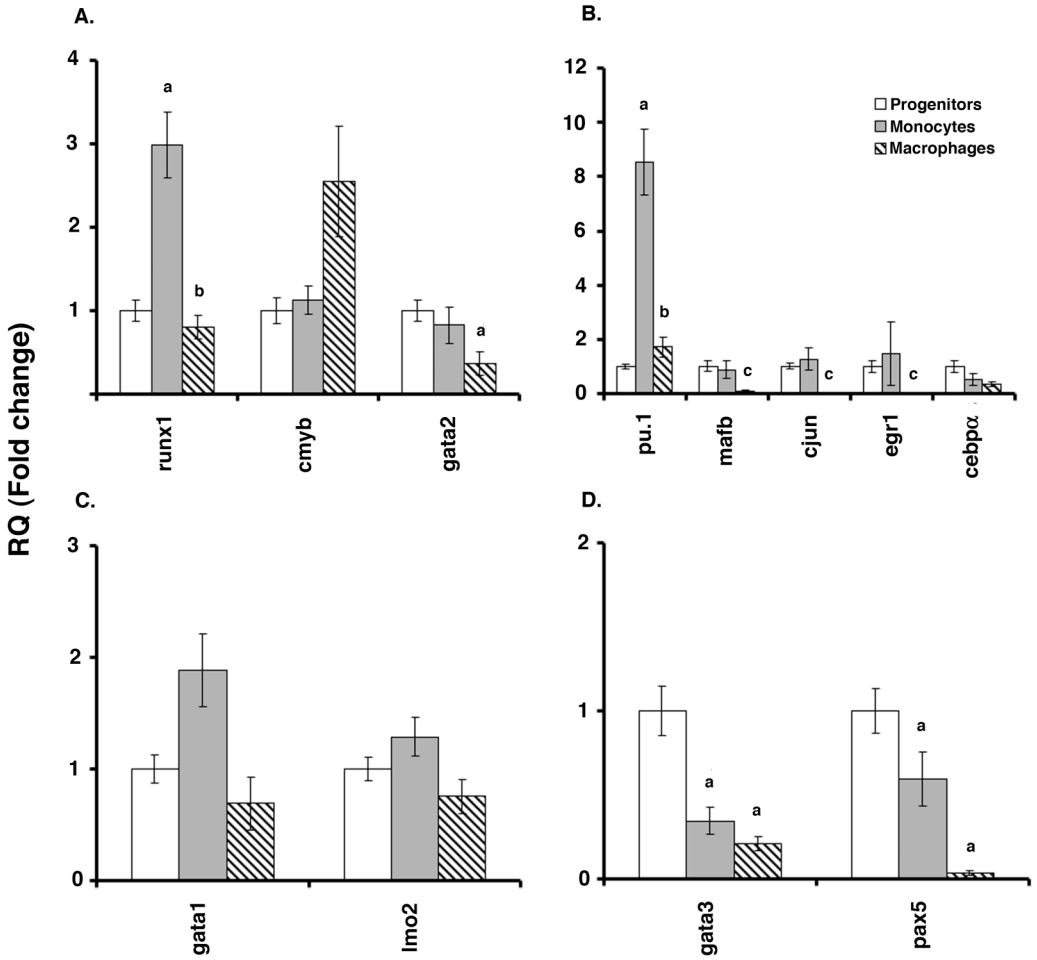


Figure 6.4: Comparison of hematopoietic transcription factor expressions during macrophage development using quantitative PCR.
Quantitative PCR was used to examine the mRNA levels of transcription factors involved in (A) early progenitor cell survival and maintenance, (B) myelopoiesis, (C) erythropoiesis and (D) lymphopoiesis in sorted progenitor cells, monocytes, and macrophages. Freshly isolated progenitor cells, monocytes from day 2-4 primary kidney macrophage cultures, or macrophages from day 6-8 primary kidney macrophage cultures were sorted from different batches of four individual fish. Significance was determined using a One-way ANOVA with a Tukey's test. Significant difference from the progenitor cell population is denoted by (a), significant difference from the monocyte population is denoted by (b), and significant difference from both the progenitor cell population and the monocyte population is denoted by (c). Significance is $P < 0.05$, ($n = 4$).

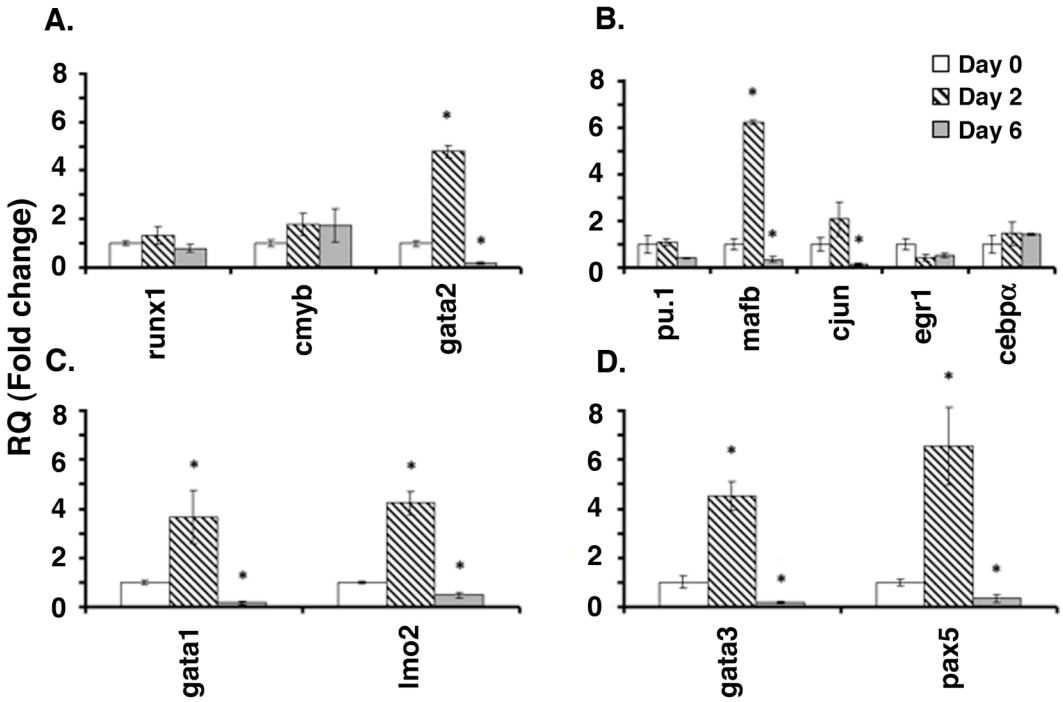


Figure 6.5: Effect of culture time on expressions of hematopoietic transcription factors by goldfish progenitor cells as determined by quantitative PCR.

Quantitative PCR expressions of transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in sorted R1 progenitor cells from Day 0, Day 2, and Day 6 PKM cultures were examined. Data was normalized to Day 0 R1 progenitor cells for each transcription factor and standard error is shown. Significance is denoted by (*) compared to the reference sample ($P < 0.05$), ($n = 3$).

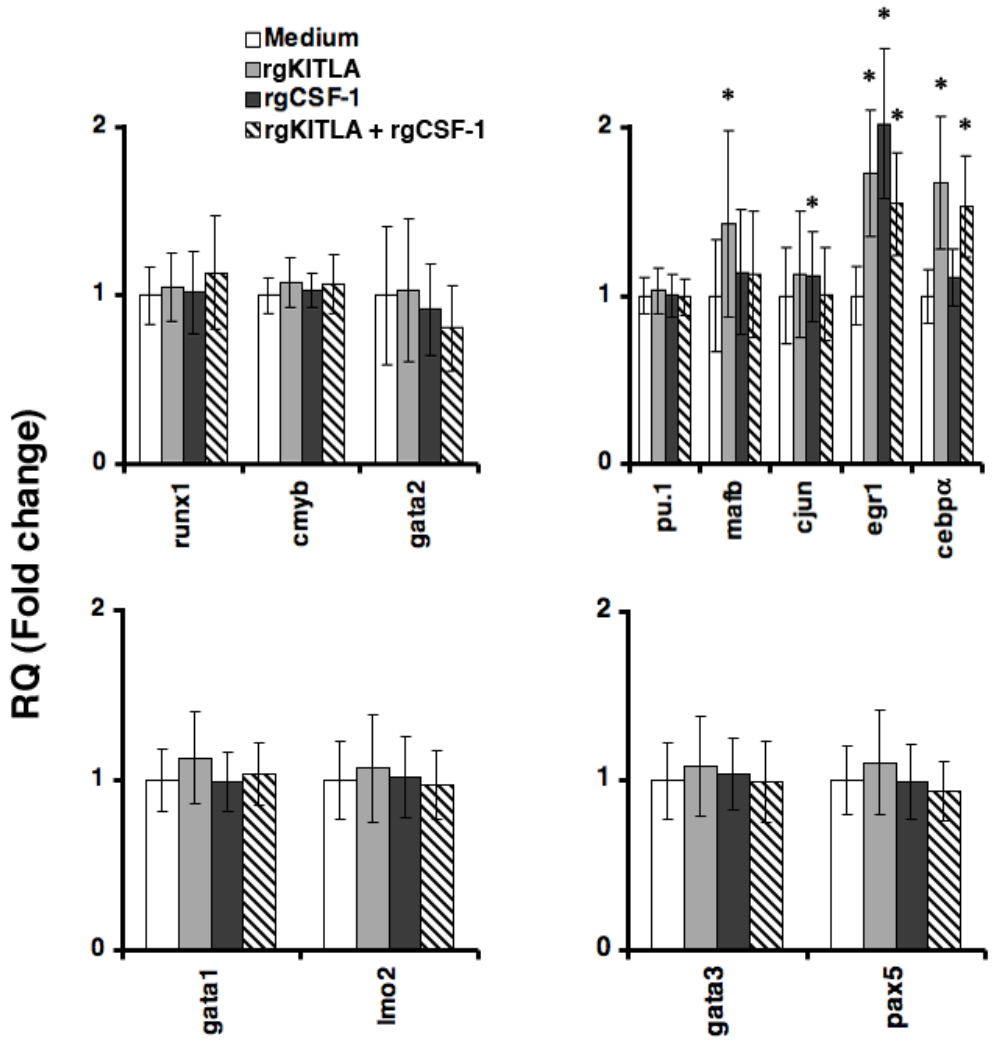


Figure 6.6: Effect of recombinant growth factors on expressions of hematopoietic transcription factors by sorted day 2 progenitor cells as determined by quantitative PCR.

Real time PCR expression of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in sorted Day 2 R1 progenitor cells treated with recombinant growth factors. R1 progenitors were seeded at a concentration of 3 x 10^5 cells/mL and treated with medium (negative control), 100 ng/mL of recombinant goldfish kit ligand a (rgKITLA), colony stimulating factor 1 (rgCSF-1), or rgKITLA and rgCSF-1 in combination for 6 hours. Data was normalized to the medium-treated R1 progenitor control cells for each transcription factor and standard error is shown. Significance is denoted by (*) compared to the reference sample ($P < 0.05$), (n = 4).

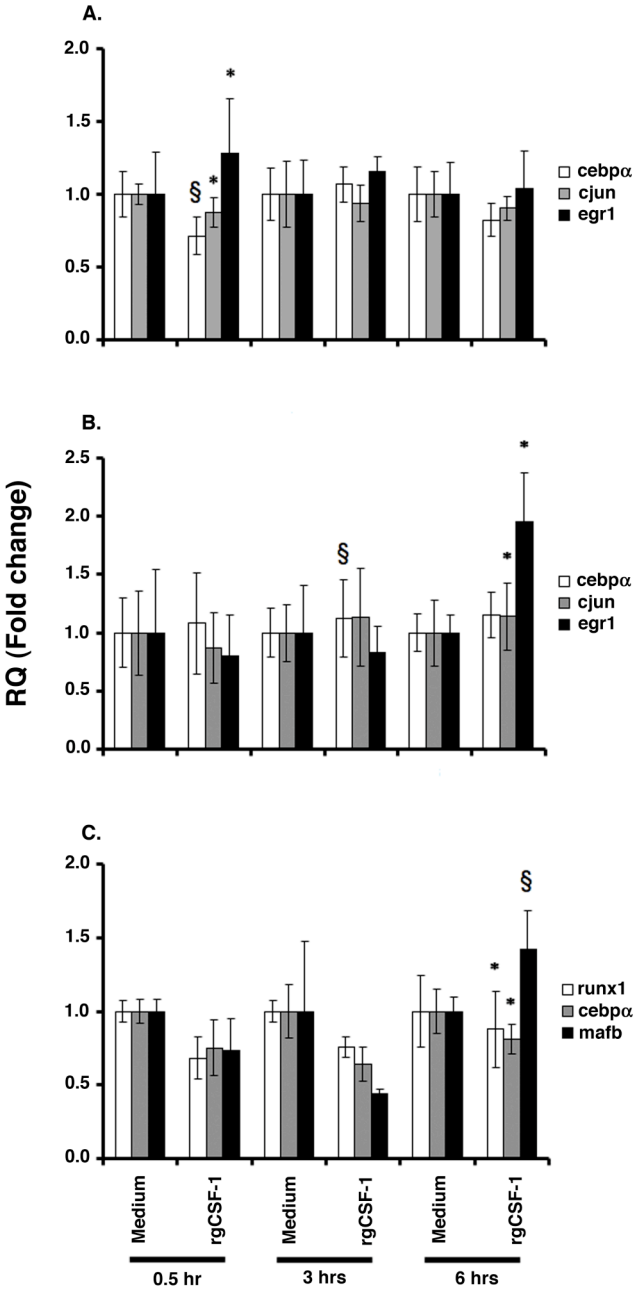


Figure 6.7: Effect of recombinant goldfish CSF-1 on myeloid transcription factor expressions in goldfish R1 progenitor cells after different days in culture as determined by quantitative PCR.
Progenitor cells from day 1 (A), day 2 (B) or day 3 (C) primary kidney macrophage cultures were sorted and treated with medium alone (negative control) or 100 ng/mL of recombinant goldfish CSF-1 (rgCSF-1) for 0.5 hr, 3 hrs, or 6 hrs prior to measuring transcription factor mRNA levels by quantitative PCR. Data were analyzed using a paired t-test. Significance is denoted by (*) to show $P < 0.05$, and (§) is $P < 0.08$, ($n = 4$).

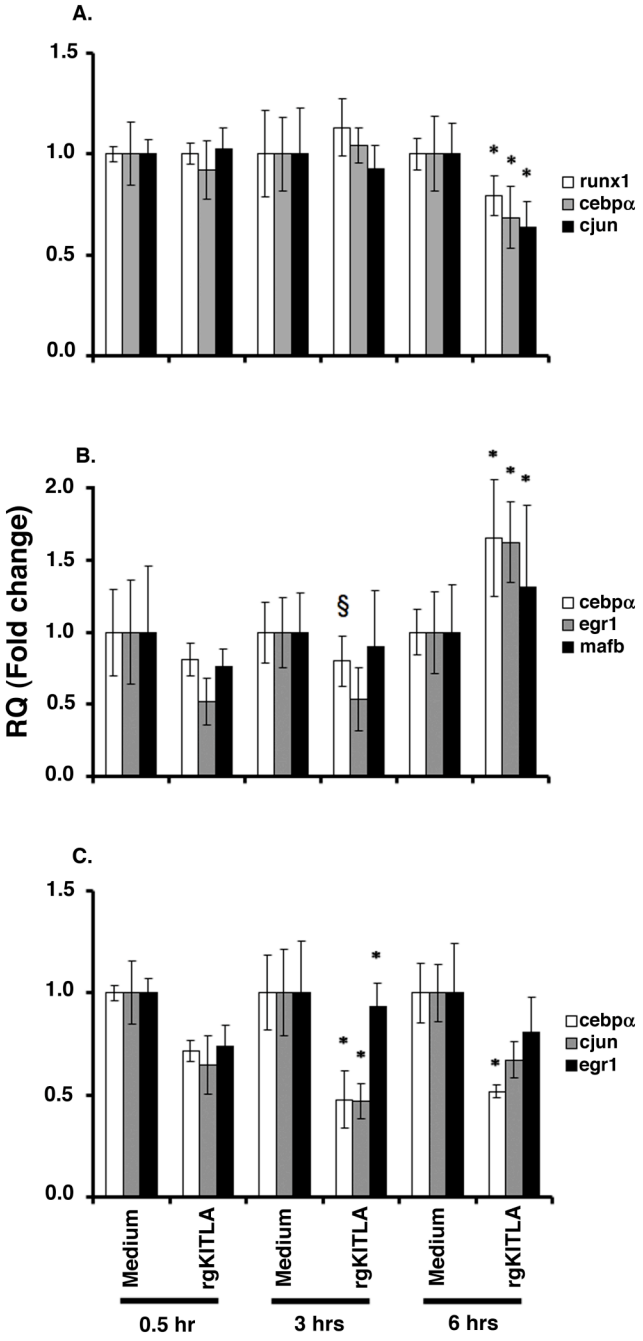


Figure 6.8: Effect of recombinant goldfish KITLA on myeloid transcription factor expressions in goldfish R1 progenitor cells after different days in culture as determined by quantitative PCR.

Progenitor cells from day 1 (A), day 2 (B) or day 3 (C) primary kidney macrophage cultures were sorted and treated with medium alone (negative control) or 100 ng/mL of rgKITLA for 0.5 hr, 3 hrs, or 6 hrs prior to measuring transcription factor mRNA levels by quantitative PCR. Data were analyzed using a paired t-test. Significance is denoted by (*) to show $P < 0.05$, and $P < 0.08$ is denoted by (§) ($n = 4$).

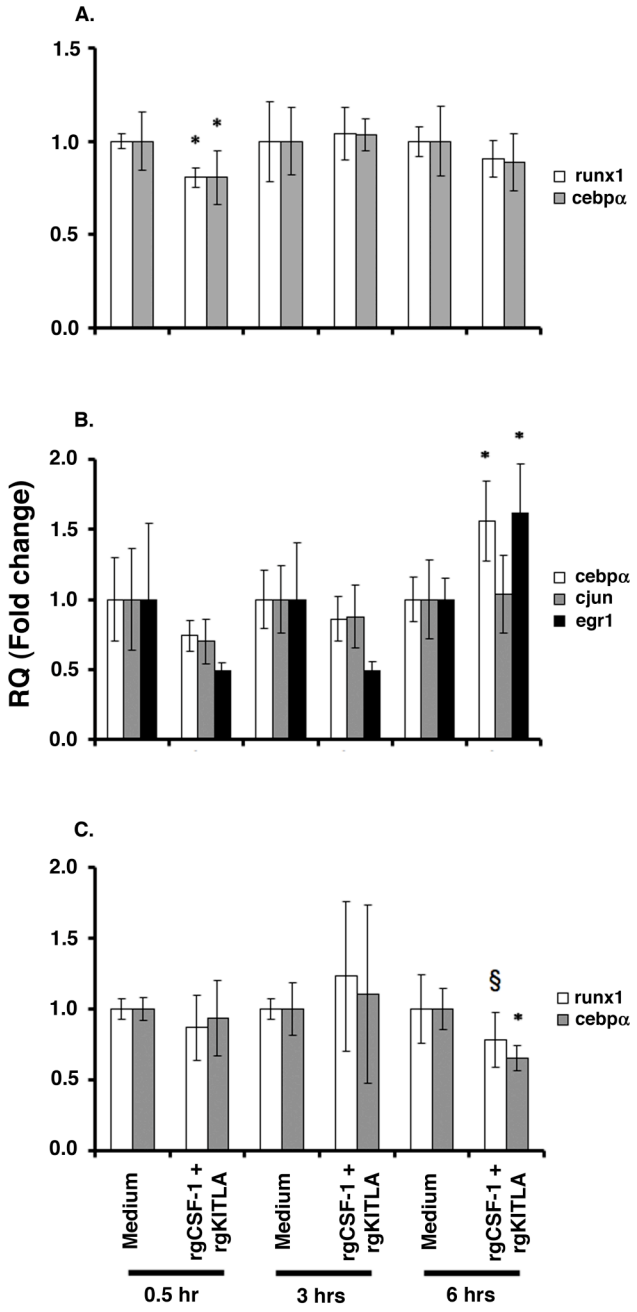


Figure 6.9: Effect of recombinant goldfish CSF-1 and KITLA on myeloid transcription factor expressions in goldfish R1 progenitor cells after different days in culture as determined by quantitative PCR.
Progenitor cells from day 1 (A), day 2 (B) or day 3 (C) primary kidney macrophage cultures were sorted and treated with medium alone (negative control) or 100 ng/mL rgCSF-1 and 100 ng/mL of rgKITLA for 0.5 hr, 3 hrs, or 6 hrs prior to measuring transcription factor mRNA levels by quantitative PCR. Data were analyzed using a paired t-test. Significance is denoted by (*) to show $P < 0.05$, and (§) is $P < 0.08$, ($n = 4$).

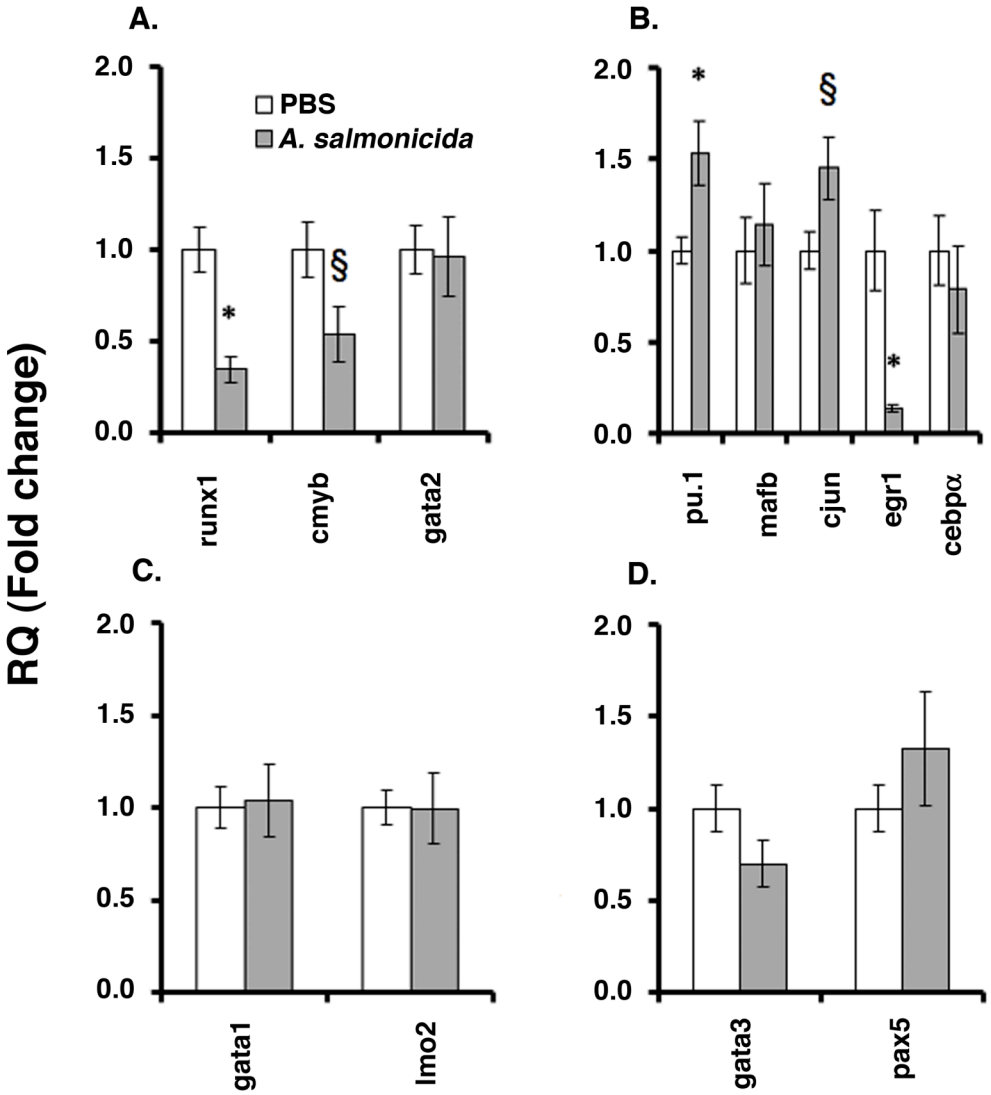


Figure 6.10: Comparison of goldfish hematopoietic transcription factors in sorted R1 progenitor cells from PBS injected or heat-killed *A. salmonicida* A449 challenged goldfish as determined by quantitative PCR.
Quantitative PCR of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in sorted R1 progenitor cells from PBS injected or heat-killed *A. salmonicida* A449 challenged fish. Fish were injected with 1 x 10^9 CFU of heat-killed *A. salmonicida* A449 or an equal volume of PBS (negative control). R1 progenitors were isolated from fish 72 hours post challenge. R1 progenitor cells were sorted from each sample to remove contaminating cells. Data were normalized to R1 progenitor cells from PBS injected fish for each transcription factor and standard error is shown. Data were analyzed using an unpaired t-test. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§), (n = 4).

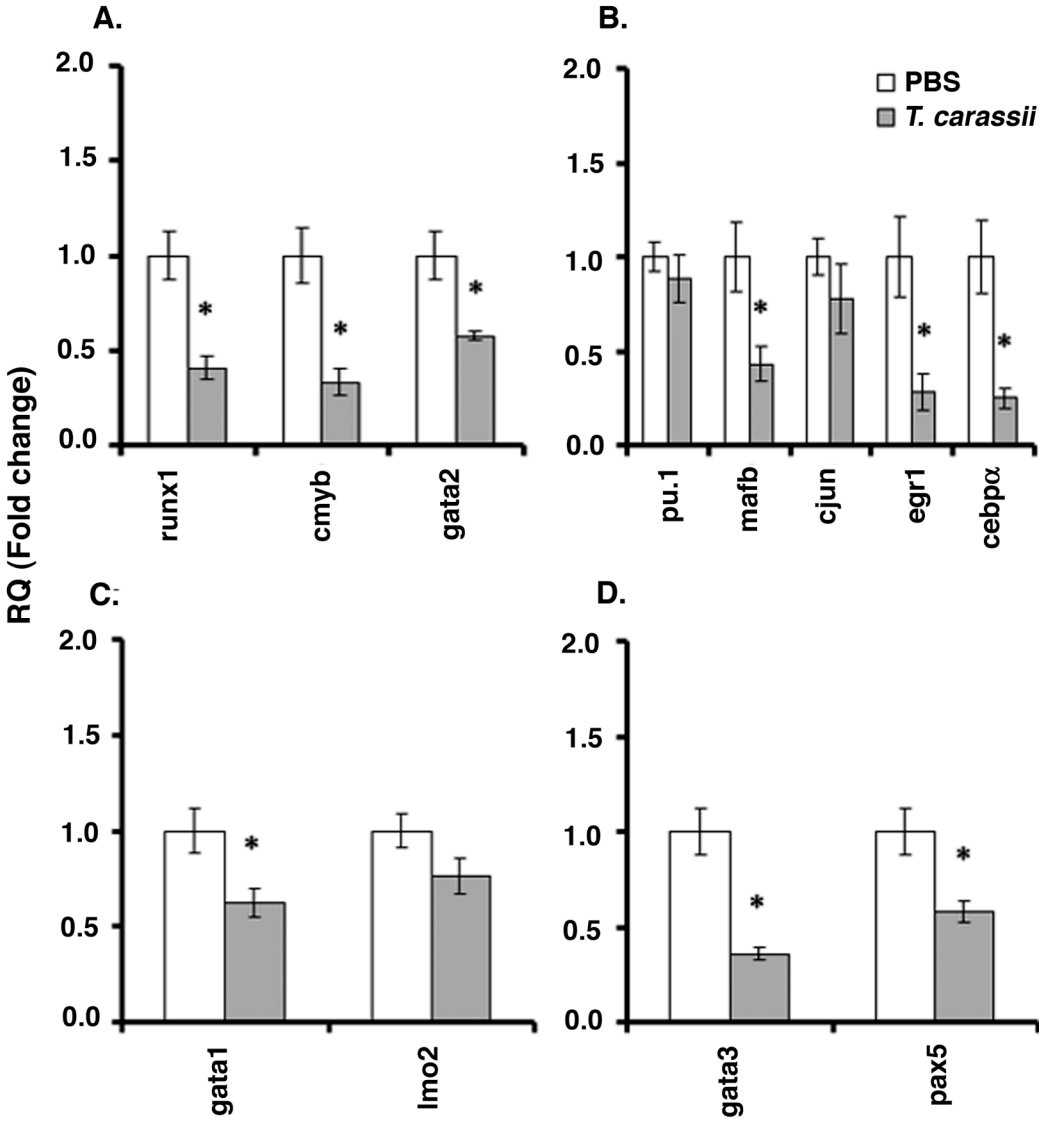


Figure 6.11: Comparison of goldfish hematopoietic transcription factor expressions in sorted R1 progenitor cells from PBS or *T. carassii* challenged goldfish as determined by quantitative PCR.
Quantitative PCR of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in sorted R1 progenitor cells from PBS or *T. carassii* challenged fish. Fish were injected with 1 x 10^8 trypanosomes or an equal volume of PBS (negative control) and R1 progenitors were isolated from fish 72 hours post challenge. Data was normalized to R1 progenitor cells from PBS injected fish for each transcription factor and standard error is shown. Data were analyzed using an unpaired t-test. Significance is denoted by (*) compared to the reference sample ($P < 0.05$), (n = 4).

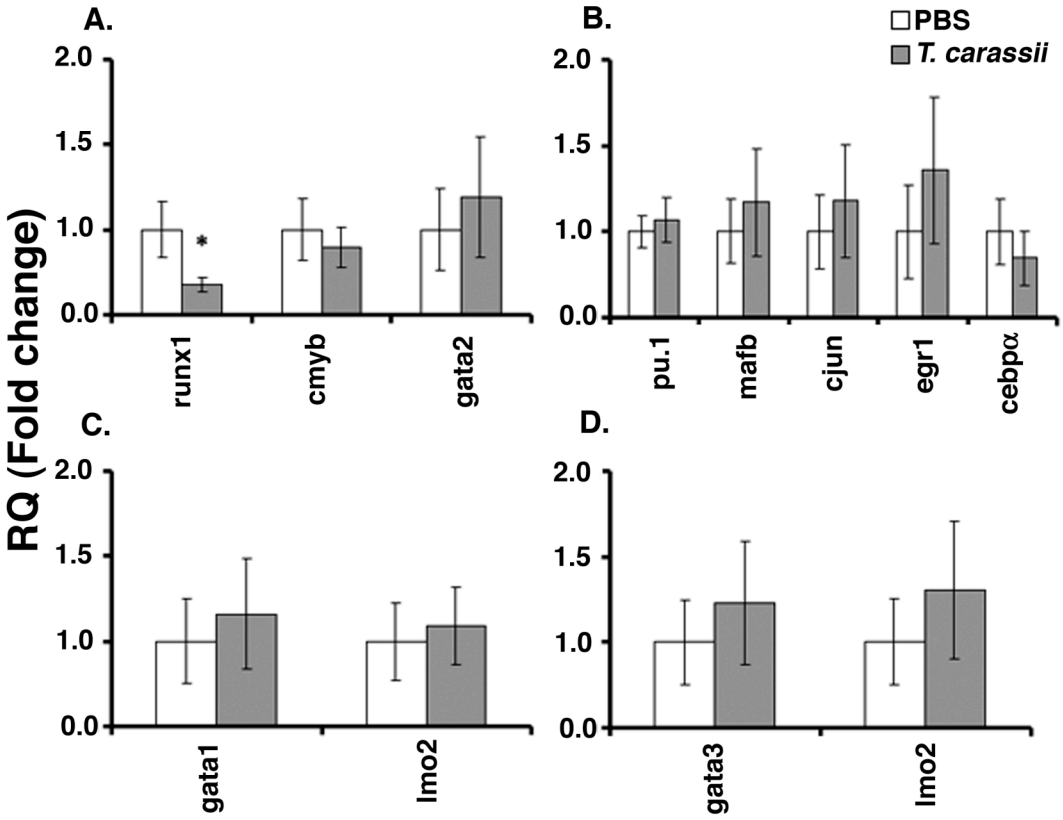


Figure 6.12: Comparison of hematopoietic transcription factor expressions in kidney tissue from PBS or *T. carassii* challenged goldfish as determined by quantitative PCR.

Quantitative PCR expressions of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in kidney tissue from PBS (control) or *T. carassii* challenged fish. Fish were injected with 6.25 x 10^6 trypanosomes or an equal volume of PBS (negative control) and kidney tissue isolated from fish 7 days post infection. Data were normalized to kidneys from PBS injected fish for each transcription factor and standard error is shown. Data were analyzed using an un-paired t-test. Significance is denoted by (*) compared to the reference sample ($P < 0.05$), ($n = 4$).

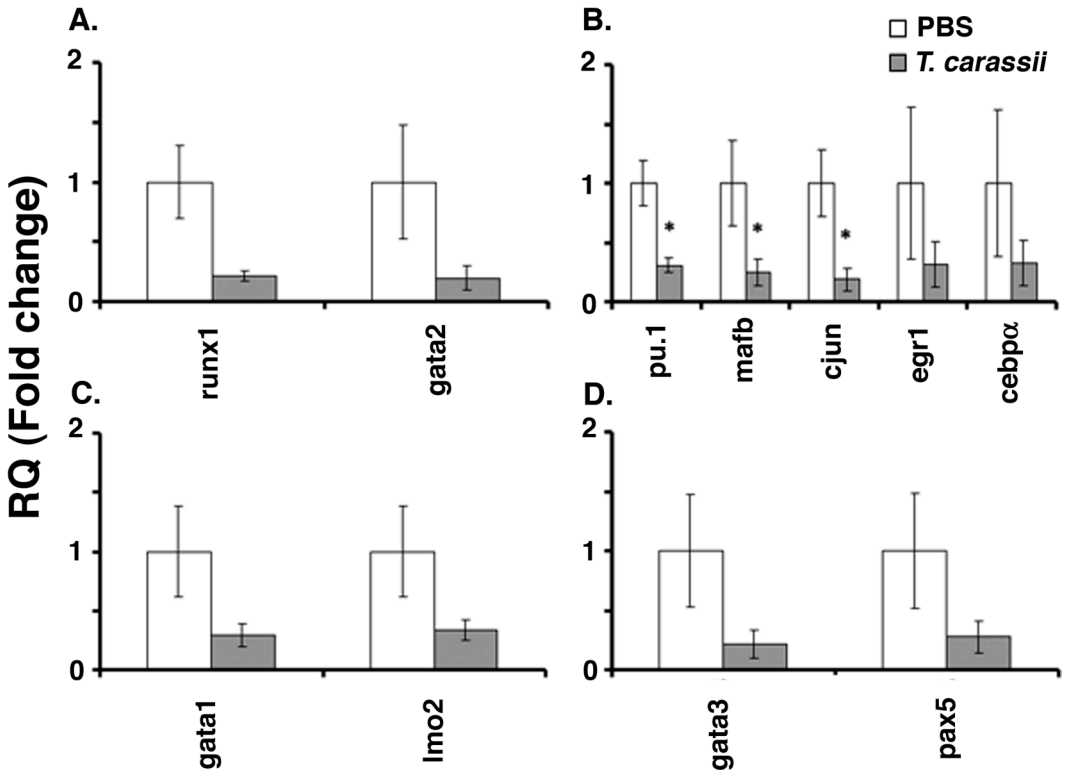


Figure 6.13: Comparison of hematopoietic transcription factor expressions in spleen tissue from PBS or *T. carassii* challenged goldfish.
Real time PCR expressions of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in spleen tissue from PBS (control) or *T. carassii* challenged fish. Fish were injected with 6.25 x 10^6 trypanosomes or an equal volume of PBS (negative control) and spleen tissue isolated from fish 7 days post infection. Data was normalized to spleens from PBS injected fish for each transcription factor and standard error is shown. Data were analyzed using an unpaired t-test. Significance is denoted by (*) compared to the reference sample ($P < 0.05$), ($n = 4$).

6.4 References:

[1] **Zhu J, Emerson SG.** Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene. 2002;21(21):3295-313.

[2] **Cantor AB, Orkin SH.** Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene. 2002;21(21):3368-76.

[3] **Friedman AD.** Transcriptional regulation of granulocyte and monocyte development. Oncogene. 2002;21(21):3377-90.

[4] **Ye M, Graf T.** Early decisions in lymphoid development. Curr Opin Immunol. 2007;19(2):123-8.

[5] **Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC, Schroeder T.** Hematopoietic cytokines can instruct lineage choice. Science. 2009;325(5937):217-8.

[6] **Metcalf D.** Lineage commitment and maturation in hematopoietic cells: the case for extrinsic regulation. Blood. 1998;92(2):345-7; discussion 52.

[7] **Metcalf D.** Hematopoietic cytokines. Blood. 2008;111(2):485-91.

[8] **Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C.** Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. Clin Cancer Res. 2008;14(7):1926-30.

[9] **Ashman LK.** The biology of stem cell factor and its receptor C-kit. Int J Biochem Cell Biol. 1999;31(10):1037-51.

[10] **Barreda DR, Hanington PC, Belosevic M.** Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol. 2004;28(5):509-54.

[11] **Panopoulos AD, Watowich SS.** Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. Cytokine. 2008;42(3):277-88.

[12] **Fliedner TM, Graessle DH.** Hematopoietic cell renewal systems: mechanisms of coping and failing after chronic exposure to ionizing radiation. Radiat Environ Biophys. 2008;47(1):63-9.

[13] **Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C.** Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of Listeria monocytogenes-infected mice. Blood. 1998;91(3):863-9.

[14] **Rieger MA, Schroeder T.** Instruction of lineage choice by hematopoietic cytokines. Cell Cycle. 2009;8(24):4019-20.

[15] **Krishnaraju K, Hoffman B, Liebermann DA.** Early growth response gene 1 stimulates development of hematopoietic progenitor cells along the macrophage

lineage at the expense of the granulocyte and erythroid lineages. Blood. 2001;97(5):1298-305.

[16] **Carter JH, Tourtellotte WG.** Early growth response transcriptional regulators are dispensable for macrophage differentiation. J Immunol. 2007;178(5):3038-47.

[17] **Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, et al.** Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell. 2006;126(4):755-66.

[18] **Zarebski A, Velu CS, Baktula AM, Bourdeau T, Horman SR, Basu S, et al.** Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. Immunity. 2008;28(3):370-80.

[19] **Nutt SL, Heavey B, Rolink AG, Busslinger M.** Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature. 1999;401(6753):556-62.

[20] **Zwollo P, Mott K, Barr M.** Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing "B cell signatures". Dev Comp Immunol. 2010;34(12):1291-9.

[21] **Hsia N, Zon LI.** Transcriptional regulation of hematopoietic stem cell development in zebrafish. Exp Hematol. 2005;33(9):1007-14.

[22] **Paik EJ, Zon LI.** Hematopoietic development in the zebrafish. Int J Dev Biol. 2010;54(6-7):1127-37.

[23] **Ellett F, Lieschke GJ.** Zebrafish as a model for vertebrate hematopoiesis. Curr Opin Pharmacol. 2010;10(5):563-70.

[24] **Loose M, Swiers G, Patient R.** Transcriptional networks regulating hematopoietic cell fate decisions. Curr Opin Hematol. 2007;14(4):307-14.

[25] **Hosoya T, Maillard I, Engel JD.** From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. Immunol Rev. 2010;238(1):110-25.

[26] **Burda P, Laslo P, Stopka T.** The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. Leukemia. 2010;24(7):1249-57.

[27] **Cai DH, Wang D, Keefer J, Yeamans C, Hensley K, Friedman AD.** C/EBP alpha:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBP alpha homodimers or AP-1. Oncogene. 2008;27(19):2772-9.

[28] **Aziz A, Soucie E, Sarrazin S, Sieweke MH.** MafB/c-Maf deficiency enables self-renewal of differentiated functional macrophages. Science. 2009;326(5954):867-71.

[29] **Sarrazin S, Mossadegh-Keller N, Fukao T, Aziz A, Mourcin F, Vanhille L, et al.** MafB restricts M-CSF-dependent myeloid commitment divisions of hematopoietic stem cells. Cell. 2009;138(2):300-13.

[30] **Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG.** Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A. 1997;94(2):569-74.

[31] **Brandt SJ, Koury MJ.** Regulation of LMO2 mRNA and protein expression in erythroid differentiation. Haematologica. 2009;94(4):447-8.

[32] **Fuxa M, Busslinger M.** Reporter gene insertions reveal a strictly B lymphoid-specific expression pattern of Pax5 in support of its B cell identity function. J Immunol. 2007;178(5):3031-7.

[33] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[34] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[35] **Hanington PC, Tam J, Katzenback BA, Hitchen SJ, Barreda DR, Belosevic M.** Development of macrophages of cyprinid fish. Dev Comp Immunol. 2009;33(4):411-29.

[36] **Barreda DR, Belosevic M.** Characterisation of growth enhancing factor production in different phases of in vitro fish macrophage development. Fish Shellfish Immunol. 2001;11(2):169-85.

[37] **Barreda DR, Hanington PC, Stafford JL, Belosevic M.** A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[38] **Barreda DR, Neumann NF, Belosevic M.** Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Dev Comp Immunol. 2000;24(4):395-406.

[39] **Hanington PC, Wang T, Secombes CJ, Belosevic M.** Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[40] **Hanington PC, Hitchen SJ, Beamish LA, Belosevic M.** Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. Fish Shellfish Immunol. 2009;26(1):1-9.

[41] **Katzenback BA, Belosevic M.** Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney. Dev Comp Immunol. 2009;33(4):601-11.

[42] **Katzenback BA, Karpman M, Belosevic M.** Distribution and expression analysis of transcription factors in tissues and progenitor cell populations of the goldfish (*Carassius auratus* L.) in response to growth factors and pathogens. Mol Immunol. 2011.

[43] **Bertrand JY, Kim AD, Teng S, Traver D.** $CD41^{+}$ $cmyb^{+}$ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. Development. 2008;135(10):1853-62.

[44] **Soza-Ried C, Hess I, Netuschil N, Schorpp M, Boehm T.** Essential role of c-myb in definitive hematopoiesis is evolutionarily conserved. Proc Natl Acad Sci U S A. 2010;107(40):17304-8.

[45] **Kobayashi I, Sekiya M, Moritomo T, Ototake M, Nakanishi T.** Demonstration of hematopoietic stem cells in ginbuna carp (*Carassius auratus* langsdorfii) kidney. Dev Comp Immunol. 2006;30(11):1034-46.

[46] **Kobayashi I, Moritomo T, Ototake M, Nakanishi T.** Isolation of side population cells from ginbuna carp (*Carassius auratus langsdorfii*) kidney hematopoietic tissues. Dev Comp Immunol. 2007;31(7):696-707.

[47] **Kobayashi I, Kuniyoshi S, Saito K, Moritomo T, Takahashi T, Nakanishi T.** Long-term hematopoietic reconstitution by transplantation of kidney hematopoietic stem cells in lethally irradiated clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Dev Comp Immunol. 2008;32(8):957-65.

[48] **Swiers G, de Bruijn M, Speck NA.** Hematopoietic stem cell emergence in the conceptus and the role of Runx1. Int J Dev Biol. 2010;54(6-7):1151-63.

[49] **Wong WF, Kohu K, Chiba T, Sato T, Satake M.** Interplay of transcription factors in T-cell differentiation and function: the role of Runx. Immunology. 2011;132(2):157-64.

[50] **Tsai FY, Orkin SH.** Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood. 1997;89(10):3636-43.

[51] **Reite OB, Evensen O.** Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish Shellfish Immunol. 2006;20(2):192-208.

[52] **Dobson JT, Seibert J, Teh EM, Da'as S, Fraser RB, Paw BH, et al.** Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. Blood. 2008;112(7):2969-72.

[53] **Aude-Garcia C, Collin-Faure V, Bausinger H, Hanau D, Rabilloud T, Lemercier C.** Dual roles for MEF2A and MEF2D during human macrophage terminal differentiation and c-Jun expression. Biochem J. 2010;430(2):237-44.

[54] **Lord KA, Abdollahi A, Hoffman-Liebermann B, Liebermann DA.** Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation. Mol Cell Biol. 1993;13(2):841-51.

[55] **Krishnaraju K, Hoffman B, Liebermann DA.** The zinc finger transcription factor Egr-1 activates macrophage differentiation in M1 myeloblastic leukemia cells. Blood. 1998;92(6):1957-66.

[56] **Kobayashi M, Yamamoto M.** Regulation of GATA1 gene expression. J Biochem. 2007;142(1):1-10.

[57] **Takizawa F, Mizunaga Y, Araki K, Moritomo T, Ototake M, Nakanishi T.** GATA3 mRNA in ginbuna crucian carp (*Carassius auratus langsdorfii*): cDNA cloning, splice variants and expression analysis. Dev Comp Immunol. 2008;32(8):898-907.

[58] **Adams B, Dorfler P, Aguzzi A, Kozmik Z, Urbanek P, Maurer-Fogy I, et al.** Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. Genes Dev. 1992;6(9):1589-607.

[59] **Akashi K, Traver D, Miyamoto T, Weissman IL.** A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404(6774):193-7.

[60] **Tagoh H, Himes R, Clarke D, Leenen PJ, Riggs AD, Hume D, et al.** Transcription factor complex formation and chromatin fine structure alterations at the murine *c-fms* (CSF-1 receptor) locus during maturation of myeloid precursor cells. Genes Dev. 2002;16(13):1721-37.

[61] **Gangenahalli GU, Gupta P, Saluja D, Verma YK, Kishore V, Chandra R, et al.** Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. Stem Cells Dev. 2005;14(2):140-52.

[62] **Liu H, Shi B, Huang CC, Eksarko P, Pope RM.** Transcriptional diversity during monocyte to macrophage differentiation. Immunol Lett. 2008;117(1):70-80.

[63] **Kobayashi I, Ono H, Moritomo T, Kano K, Nakanishi T, Suda T.** Comparative gene expression analysis of zebrafish and mammals identifies common regulators in hematopoietic stem cells. Blood. 2010;115(2):e1-9.

[64] **Maeda K, Nishiyama C, Ogawa H, Okumura K.** GATA2 and Sp1 positively regulate the c-kit promoter in mast cells. J Immunol. 2010;185(7):4252-60.

[65] **Nguyen HQ, Hoffman-Liebermann B, Liebermann DA.** The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell. 1993;72(2):197-209.

[66] **Hanington PC, Belosevic M.** Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. Dev Comp Immunol. 2007;31(8):817-29.

[67] **Hanington PC, Brennan LJ, Belosevic M, Andrew Keddie B.** Molecular and functional characterization of granulin-like molecules of insects. Insect Biochem Mol Biol. 2008;38(5):596-603.

[68] **Katzenback BA, Belosevic M.** Molecular and functional characterization of kita and kitla of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2009;33(11):1165-75.

[69] **DeLigio JT, Zorio DA.** Early growth response 1 (EGR1): a gene with as many names as biological functions. Cancer Biol Ther. 2009;8(20):1889-92.

[70] **Kondo M.** Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. Immunol Rev. 2010;238(1):37-46.

[71] **Serbina NV, Hohl TM, Cherny M, Pamer EG.** Selective expansion of the monocytic lineage directed by bacterial infection. J Immunol. 2009;183(3):1900-10.

[72] **Clayton CE, Selkirk ME, Corsini CA, Ogilvie BM, Askonas BA.** Murine trypanosomiasis: cellular proliferation and functional depletion in the blood,

peritoneum, and spleen related to changes in bone marrow stem cells. Infect Immun. 1980;28(3):824-31.

[73] **Gibbons AE, Price P, Shellam GR.** Analysis of hematopoietic stem and progenitor cell populations in cytomegalovirus-infected mice. Blood. 1995;86(2):473-81.

Chapter 7: Isolation and functional characterization of goldfish (*Carassius auratus* L.) kidney neutrophils[4]

7.1 Introduction

Neutrophils are a key component of the immune response against a variety of bacterial, viral, protozoan and fungal pathogens. As one of the first cells recruited to the inflammatory site, neutrophils possess a formidable armamentarium of responses that can, in most cases, efficiently remove the offending pathogens. Neutrophils can phagocytose [1], produce toxic reactive oxygen intermediates, degranulate and send out neutrophil extracellular traps (NETs) in response to invading pathogens [2-6]. While mammalian neutrophils have been extensively characterized, relatively little is known about their teleost counterparts, and to date, the isolation and functional characterization of goldfish (*Carassius auratus* L.) neutrophils has not been reported.

The classification of fish leukocytes has been based on cytochemical staining characteristics of mammalian cells leading to the designation of fish granulocytes as acidophilic, basophilic and neutrophilic [7, 8]. However, granulocytes of many fish species do not fit into such a rigid classification scheme as some fish lack basophil-like, or eosinophil-like cells, while others, such as carp and goldfish appear to possess neutrophil-like cells, and a granulocytic cell that have staining characteristics of both basophils and eosinophils [7]. In some teleosts the neutrophilic or heterophilic granulocytes

[4] A version of this chapter has been published. Katzenback and Belosevic, 2009. *Developmental and Comparative Immunology* 33:601-611.

have been found to be comparable to mammalian neutrophils, as they also possess the capacity to perform such functions as chemotaxis [9], phagocytosis [10], respiratory burst [11], degranulation [12], and the ability to send out NETs [13, 14]. However, the varying cytochemical characteristics between different fish species may suggest that these “neutrophil-like” cells may be functionally diverse in different fish species as exemplified by the spontaneous cytotoxicity of carp neutrophils toward human target cells [15]. Methods to accurately assess the fish neutrophil cell armamentarium in response to pathogens such as chemotaxis, production of reactive oxygen intermediates, degranulation of primary granules, and antimicrobial activity have been adapted from those developed in mammals [12, 16-19].

The isolation and characterization of granulocytes in lower vertebrates is important for understanding mechanisms of host innate immune responses against fish pathogens, and the range of antimicrobial mechanisms that neutrophils posses underscores their importance in host defense. In an aquacultural setting, due to crowded conditions and increased stress, fish are highly susceptible to pathogens such as *Aeromonas salmonicida*, a gram-negative bacterium, which is the causative agent of furunculosis in salmonids, erythrodermatitis in carp and ulcerative disease in goldfish [20, 21].

Studies examining this infectious disease in salmonids have focused on macrophage-*Aeromonas* interactions; however, macrophages are not very effective at killing virulent strains of *Aeromonas* spp., which may be due to the toxicity effects of the bacteria towards the macrophages [22, 23]. Studies on Atlantic salmon neutrophil-*Aeromonas* interactions show that neutrophils migrate, phagocytose, and produce a respiratory burst response when exposed to the

bacteria *in vitro* [24, 25]. The functional assessment of neutrophils in cyprinids have shown that carp neutrophils can mount a significant respiratory burst response and are able to kill *Aeromonas in vitro*, despite the fact that some toxic effects on neutrophils were observed when high numbers of bacteria were added to the neutrophil cultures [26, 27].

This chapter describes the isolation and cytochemical and functional characterization of goldfish neutrophils. To date, the isolation of a large number of relatively pure goldfish neutrophils has not been reported. Specifically, I show that goldfish kidney neutrophils represent a population of cells that are capable of mounting antimicrobial responses when treated with mitogens or pathogens. The capacity of the kidney neutrophil to respond to stimuli suggest that they are a mature population of cells that could be called upon to efficiently participate in host defense against invading pathogens. The objectives of the experiments described in this chapter were to (1) isolate a population of goldfish neutrophils from the kidney, (2) cytochemically characterize the kidney neutrophils, and (3) perform a comprehensive functional assessment of goldfish neutrophils in response to mitogens and to the virulent fish pathogen, *Aeromonas salmonicida* strain A449.

7.2 Results

7.2.1 Cytochemistry

Cells isolated from the kidney of goldfish as previously detailed in chapter 3 were greater than 99% viable based on trypan blue staining, were non-adherent in culture, and resembled neutrophils based on their cytochemical

staining. Cells stained with hematoxylin/eosin were 10-12 μm in size with a round morphology. Their nuclei were round or indented and they had clear granulocytic cytoplasm (Fig. 7.1A). The un-segmented nature of the neutrophil cell nuclei is characteristic of myelocyte and metamyelocyte neutrophils, or heterophil cells observed in the kidney of other teleosts such as the zebrafish [28], and is in agreement with the hematopoietic nature of this organ in fish. Upon maturation these neutrophils, or heterophils as they are often called, develop a segmented or multi-lobed nucleus [28, 29]. The goldfish neutrophilic cells stain positive with Sudan Black (Fig 7.1B), myeloperoxidase (Fig. 7.1C), acid phosphatase (Fig 7.1E) that was tartrate-sensitive (not shown). These cells were slightly positive for Periodic Acid Schiff (Fig. 7.1F), but were negative for non-specific esterase (Fig 7.1D). Sudan Black staining has been used as a key indicator of mammalian neutrophils, and based on Sudan Black staining the neutrophil cell preparations were of high purity (>92%). Additionally, the negative non-specific esterase staining characteristics of these neutrophils indicated that they were not monocytes/macrophages. The cytochemical staining characteristics of goldfish neutrophils were similar to that observed for neutrophils of other teleosts.

Immunofluorescence labeling using a polyclonal antibody towards soluble colony-stimulating factor-1 receptor (sCSF-1R) was also performed as previously described [30] and indicated the absence of surface CSF-1R on 91% of the neutrophils (data not shown, over 600 cells observed in 10 random fields of view using a fluorescent microscope). This was consistent with the purity of the neutrophil population, as indicated by Sudan Black staining, and suggests that this subpopulation of leukocytes contained a relatively pure population of neutrophils.

7.2.2 The expression of different genes in neutrophils

To further confirm the identity of the isolated neutrophils, RT-PCR was employed to examine the expressions of myeloperoxidase and CSF-1R. For this experiment, cDNA was generated from goldfish kidney tissue, day 10 PKMs, or from an overnight culture of neutrophils pooled from 5 fish. Duplicate experiments were performed to ensure expression was representative of goldfish neutrophils. Myeloperoxidase (MPO) is an enzyme found in high abundance within the primary granules of neutrophils, and has been identified in fish neutrophils [31]. Primers for goldfish *mpo* were designed based on the sequence for zebrafish *mpo* (accession number AF349034). Additionally, to demonstrate that these leukocytes were neutrophils and not part of the monocyte/macrophage lineage, primers were used to test for the presence of *csf-1r* message. Based on RT-PCR (Fig. 7.2), kidney tissue (K) and neutrophils (N) expressed *mpo* whereas macrophages (M) did not after 26 or 36 cycles. The expression of *mpo* was consistent with the positive cytochemical reaction of neutrophils for myeloperoxidase (Fig. 7.1C). Additionally, *csf-1r* message was observed in kidney tissue and a faint band was observed in macrophages after 26 cycles. Following 36 cycles, prominent bands were observed for *csf-1r* in both kidney and macrophages and a faint band was observed in the neutrophil sample. However, I believe this small amount of *csf-1r* expression was due to the small percentage of contaminating monocytes/macrophages in the neutrophil subpopulation, as seen with immunofluorescent imaging. Consequently, I believe

that *cs-1r* is not expressed by kidney neutrophils. Based on these cytochemical and immunocytochemistry data, these cells appeared to be goldfish neutrophils.

7.2.3 Isolation and cytochemical staining of neutrophils from the blood and spleen

Due to the immature nature of the cell morphology observed with kidney neutrophils, attempts were made to isolate neutrophils from the blood and spleen of goldfish. The first attempt at using a similar protocol in which the cell suspension was layered over 51% Percoll to isolate kidney neutrophils was unsuccessful due to the large number of contaminating red blood cells. After red blood cell lysis, the large amount of membranes left behind proved problematic for recovering the remaining viable cells. Therefore, a new method was employed in which blood and spleen cells were layered over a 51%-60%-75% discontinuous Percoll gradient. Upon isolation of cells from each of these interfaces, and lysis of residual red blood cells, cells from each layer were stained with Sudan Black. The largest number of Sudan Black positive cells was observed at the interface between the 60% and 75% Percoll layers. Cell suspensions from the kidney were used as a positive control. After examination of the Sudan Black positive cells from the blood and spleen of three individual fish, cells were observed to have a similar morphology to that of cells isolated from goldfish kidney (Fig 7.1). In addition, low neutrophil purity was obtained; 7% neutrophils in the blood and 3% neutrophils in the spleen, compared to the 92% purity of neutrophils from the kidney. The low number of neutrophils obtained from the blood was expected, as a number of fish species are known to have low

numbers of circulating neutrophils in their blood [32]. The low yield and purity of neutrophils from the blood and spleen precluded any molecular or functional work that could be performed to characterize these populations of neutrophils.

7.2.4 Neutrophil degranulation induced by mitogens

One of the functional hallmarks of neutrophils, and granulocytes in general, compared to the cells of the mononuclear phagocyte lineage is their ability to degranulate. Therefore, a degranulation assay was set up to determine whether these cells had the capacity to degranulate in response to mitogens. For this experiment, neutrophils from five to six fish were pooled, and seeded in duplicate wells per treatment. The assay was repeated twice more, for a total of three independent replicates. Neutrophils from overnight cultures were re-suspended in HBSS without calcium and magnesium or in HBSS with calcium and magnesium. In the absence of calcium and magnesium neutrophils did not degranulate in the presence of any of the mitogens tested (Fig. 7.3A). This is not surprising, as it is well known that mammalian neutrophils require the presence of calcium in order to degranulate. Following the addition of calcium and magnesium, Iobserved a strong neutrophil degranulation response in the presence of calcium ionophore (CaI) and cytochalasin B as well as CaI alone (Fig. 7.3B). Following stimulation of neutrophils with CaI plus cytochalasin B, a significant release of peroxidase was observed after only 10 minutes of stimulation compared to treatment matched controls, whereas in the absence of cytochalasin B a significant amount of peroxidase was released after 20 minutes of stimulation with CaI ($P<0.05$). PMA also induced a significant but modest

degranulation of neutrophils in the presence of cytochalasin B after 20 minutes, however, there was a minimal degranulation response towards PMA alone that was only significant after 30 minutes of stimulation (Fig. 7.3B). As expected, zymosan induced a strong degranulation response only in the absence of cytochalasin B after 30 minutes of stimulation. The degranulation response of neutrophils to zymosan in the presence of cytochalasin B was not significantly different from controls at any time point (Fig. 7.3B). Based on these results, a stimulation time of thirty minutes was selected for all future degranulation assays.

I next examined whether the neutrophil degranulation response was dose-dependent. Neutrophils, pooled from 5-6 fish, were incubated overnight, and suspension cells used in the degranulation and flow cytometry assays. For the degranulation assay, duplicate wells were set up for each treatment, and four independent experiments were performed. For the flow cytometry assay, three independent experiments were conducted. CaI in the presence or absence of cytochalasin B induced a dose-dependent degranulation response of goldfish neutrophils (Fig. 7.4A). However, CaI plus cytochalasin B induced a more robust degranulation at all concentrations tested compared to that induced by CaI alone (Fig. 7.4A). The ability of CaI to induce degranulation was further supported by flow cytometry analysis. Stimulation of neutrophils with increasing concentrations of CaI, caused a decrease in both forward and side scatter parameters that are indicative of decrease in size as well as internal complexity of the cells (Fig 7.5A). Similarly, zymosan triggered degranulation measured by the total peroxidase released by neutrophils (Fig 7.4C), as well as a decrease in size and internal complexity of the cells (Fig 7.5C). This was observed only at the highest zymosan concentration tested. In contrast, PMA induced a modest degranulation

response that was only observed in the presence of cytochalasin B (Fig 7.4B). Consistent with the minimal peroxidase release observed when neutrophils were treated with PMA only, there was no apparent shift in size or internal complexity of the cells (Fig 7.5B), when analyzed by flow cytometry, in the absence of cytochalasin B. Clearly, goldfish neutrophils are able to degranulate in a time and dose-dependent manner.

7.2.5 Neutrophil degranulation induced by *A. salmonicida*

To test whether neutrophils were activated to degranulate following exposure to a pathogen, Ichose to perform a degranulation assay using the fish pathogen, *A. salmonicida* A449. Neutrophils, pooled from 5-6 fish, were cultured overnight, and suspension cells seeded into duplicate wells per treatment. Three independent replicates were performed. Neutrophils in HBSS with calcium and magnesium were treated with differing concentrations of live *A. salmonicida* corresponding to *A. salmonicida* to neutrophil ratios of 0.1:1 – 50:1. CaI in the presence of cytochalasin B was used as a positive control to ensure neutrophils were viable and capable of responding. *A. salmonicida* caused a relatively modest degranulation response at high ratios of bacteria to neutrophils (50:1 and 10:1), with a higher degranulation response observed in the presence of cytochalasin B (Fig 7.6). This suggests that neutrophils are capable of degranulation in response to this bacterium, albeit at a relatively low level.

7.2.6 Neutrophil respiratory burst response induced by mitogens

The respiratory burst response of neutrophils was employed as another means of measuring the activation of goldfish neutrophils. This was done using a flow cytometry based assay that utilized the non-fluorescent compound dihydrorhodamine (DHR) that then oxidizes to the green fluorescent rhodamine 123 in the presence of hydrogen peroxide, a product of the respiratory burst. For this experiment, neutrophils, pooled from 5-6 fish, were cultured overnight and suspension cells used in the assay. Three replicates of this experiment were performed. Neutrophils were incubated with medium alone to account for the non-specific activation of the cells due to handling (negative control) (Fig 7.7A, 7.8A, 7.9A, left panel). Based on this negative control, the M1 gate was created to measure the increasing shift in fluorescence intensity that is normally associated with a respiratory burst response. To ensure that neutrophils were being loaded properly and that the DHR reagent was functional, hydrogen peroxide was added as a positive control. As expected, the addition of hydrogen peroxide the fluorescence intensity increased (shift to the right) and into the M1 gate (Fig. 7.7A, 7.8A, 7.9A, right panel).

After 30 minutes of incubation of goldfish neutrophils in the presence of CaI, a small respiratory burst response was observed at CaI concentrations of 1 μg/mL and 10 μg/mL (Fig 7.7B, upper panels). After 60 minutes a respiratory burst response was evident at all CaI concentrations tested (Fig. 7.7B, lower panels). However, a relatively low number of intact neutrophils were measured due to the intense degranulation response that the neutrophils undergo in response to CaI (Fig. 7.5A). For this reason, there were fewer cells available to

undergo respiratory burst. Stimulation of neutrophils with PMA resulted in an intense respiratory burst response at both 30 and 60 minutes at all concentrations of PMA tested (0.1 μg/mL, 1 μg/mL and 10 μg/mL) (Fig 7.8B, upper and lower panels). In contrast, zymosan did not induce a respiratory burst response at any concentration or incubation time (Fig 7.9B). Similarity, a respiratory burst response was not observed following treatment of neutrophils with bacterial lipopolysaccharide (data not shown).

7.2.7 Neutrophil respiratory burst response induced by *A. salmonicida*

The induction of neutrophil activation by *A. salmonicida* was also determined by measuring the respiratory burst response using the DHR reagent. Neutrophils, pooled from 5-6 fish, were cultured overnight, and suspension cells used for the assay. Two independent experiments were performed. This respiratory burst assay was more complex to perform due to the fact that *A. salmonicida* had minimal auto-fluorescence, and a slight shift in fluorescence when loaded with DHR, as indicated by flow cytometry analysis. However, compared to DHR loaded neutrophils, bacterial fluorescence was minimal, and only very slightly overlapped with that of the loaded neutrophils. Nevertheless, Iexcluded all *A. salmonicida* specific fluorescence from the measurements of the respiratory burst response of neutrophils by creating a cytometer gate, R6. In all experiments, less than 3% of the entire population of neutrophils were excluded from the final measurement of the neutrophil respiratory burst response. Based on previous results, 1 μg/mL PMA was chosen as a positive control, and following treatment with PMA, approximately 72% of the neutrophils produced an

intense respiratory burst response shown in the M1 gate (Fig 7.10). When neutrophils and *A. salmonicida* were mixed together at different ratios and allowed to incubate for 1 hour prior to flow cytometry analysis, Iobserved a significantly higher neutrophil respiratory burst response at bacteria to neutrophil ratios greater than 5 bacteria to 1 neutrophil. Not surprisingly, the highest respiratory burst response was observed at ratios of 50 bacteria: 1 neutrophil, where greater than 50% of the neutrophils were activated (Fig. 7.10).

7.2.8 Neutrophil respiratory burst response over time of cultivation

To investigate the effects of cultivation on the ability of goldfish neutrophils to become activated, the measurement of the neutrophil respiratory burst responses to 0.1 μg/mL, 1 μg/mL and 10 μg/mL of PMA were chosen. Neutrophils, from 5 fish were pooled, and cultured for 0, 24, 48, 72 or 96 hours. Suspension cells were used for the assay. The neutrophil respiratory burst responses were similar between all concentrations regardless of the time point (Table 7.1). In addition, the percentage of responding cells was similar at 0 and 24 hours after isolation, however, the percentage of responding cells drastically decreased at 48 hours post isolation and continued to decrease over the 96-hour cultivation period (Table 7.1).

7.2.9 Neutrophils produce a small amount of nitric oxide in response to heat-killed *A. salmonicida*

To investigate the ability of neutrophils to produce a nitric oxide response, neutrophils were incubated with heat-killed *A. salmonicida* A449 for 48 or 72 hours prior to nitrate measurement. Neutrophils were obtained from four

individual fish, cultured overnight, and seeded in duplicate wells per treatment. At 48 and 72 hours, there was an increase in nitrate production in supernatants from neutrophils treated with bacteria compared to the time-matched medium control, although not significant (Fig. 7.11). Due to the small percentage of contaminating monocytes/macrophages within our neutrophil preparations, Icannot directly attribute the small increase in nitrate production to neutrophils. Rather, the small population of contaminating monocytes/macrophages may be responsible for the nitric oxide response observed.

7.2.10 Live *A. salmonicida* and bacteria-conditioned supernatants induced chemotactic response of neutrophils

For both sets of chemotaxis assays, neutrophils were pooled from 5-6 fish, and cultured overnight. Duplicate chemotaxis chambers were set up per treatment, and two independent experiments performed. In the presence of fMLP, a known chemoattractant, neutrophils migrated towards the source and this migration was statistically significant compared to the negative control of medium alone ($P<0.05$; one-way ANOVA) (Fig. 7.12). When live bacteria were placed in the lower chemotaxis chamber, neutrophils migrated towards the bacteria at higher concentrations (10^6 and 10^7), however, chemotaxis did not occur when lower concentrations of *A. salmonicida* were used (Fig.7.12). Furthermore, this was not random migration as the chemokinesis control, consisting of 10^6 bacteria in the upper and lower chambers of the blind-well chemotaxis apparatus, was not significantly different from the medium alone control. To further investigate the chemotactic nature of *A. salmonicida*,

Iexamined the chemotactic response of neutrophils towards bacterial lipopolysaccharide (LPS). As shown in Fig. 7.12, LPS at all concentrations tested did not induce a chemotactic response of neutrophils. In contrast, bacteria-conditioned supernatants either undiluted and diluted up to 8 times induced a statistically significant chemotactic response of goldfish neutrophils compared to medium alone control (Fig. 7.13). The chemotactic response was directional migration rather than random migration as indicated by non-significant chemokinetic response results (Fig. 7.13). While undiluted supernatants were highly chemotactic (~200 neutrophils/ 20 fields of view) (Fig. 7.13), they did not induce as high of a chemotactic response as the live bacteria (~325 neutrophils/ 20 fields of view) (Fig. 7.11).

7.3 Discussion

During an inflammatory response neutrophils are essential for clearance of pathogens and recruitment of additional cells to the inflammatory sites. I demonstrated that a relatively pure neutrophil preparations can be obtained from goldfish kidney, based on Sudan Black staining and lack of immunofluorescence labeling with an anti-sCSF-1 receptor antibody. These cells have characteristic nuclear morphology of neutrophil myelocyte and metamyelocyte developmental stages [28], which was consistent with the hematopoietic nature of the fish kidney. The morphology and cytochemical staining of goldfish kidney neutrophils was similar to neutrophils from other teleost groups [7]. Due to the round or indented shape of the neutrophil nucleus, these cells looked similar to monocytes. To ensure these cells were indeed neutrophils in nature, I performed

RT-PCR for both myeloperoxidase and CSF-1 receptor message. I chose myeloperoxidase as a marker as both mammalian, and recently fish myeloperoxidase, has been used as a marker of neutrophils as it is contained within the primary granules of neutrophils. Additionally, the CSF-1 receptor is involved in the development of macrophages in mammals and fish, and thus should not be expressed in neutrophils. In addition to cytochemical staining, I employed immunofluorescent staining using sCSF-1 receptor antibody, previously developed in the Belosevic laboratory [30]. Based on these results, I believe these cells to be neutrophils and undertook functional studies to fully characterize these cells.

After treatment with Ca^{2+} ionophore, PMA, zymosan, or live *A. salmonicida*, goldfish neutrophils exhibited dose-dependent degranulation within thirty minutes of stimulation in the presence of calcium and magnesium. CaI with cytochalasin B produced the strongest degranulation response out of all the mitogens tested, whereas PMA only induced degranulation in the presence of cytochalasin B and zymosan in the absence of cytochalasin B. These results are similar to those reported for fathead minnows [12]. However, goldfish neutrophils did not release as much peroxidase when compared to fathead minnow neutrophils, 60% and 40% for fathead minnow and goldfish, respectively. This was not unexpected since differences in activation of neutrophils from different bony fish have been reported [33]. The presence of cytochalasin B for induction of degranulation (although the mechanism of action has yet to be fully addressed in fish) was shown to be required for both mammalian and fish neutrophils [12, 18, 34]. The surprising observation in the present study was that I observed a degranulation response following treatment of neutrophils with CaI in the

absence of cytochalasin B or following treatment of the cells with PMA and cytochalasin B. This is in contrast to what was observed for bovine neutrophils, where degranulation was not induced by PMA in the presence or absence of cytochalasin B, or by CaI in the absence of cytochalasin B [18].

Neutrophils possess both oxygen independent and oxygen dependent mechanisms for destruction of pathogens. In particular, when activated these cells produce copious amounts of reactive oxygen intermediates. I demonstrated in this study that goldfish neutrophils were capable of a significant respiratory burst response that was induced either by different mitogen combinations or *live A. salmonicida.* While PMA did not induce a strong degranulation response, it triggered an intense respiratory burst response of neutrophils that was consistent with what has been reported for neutrophils of other teleost groups [26, 27, 35]. Although CaI also induced a respiratory burst response of neutrophils, fewer cells exhibited this response which I believe was due to intense degranulation of the cells induced by this reagent, even in the absence of cytochalasin B.

In both the degranulation and respiratory burst assays there was a small population of cells that did not respond to the various treatments. Based on cytochemical staining and immunofluorescence staining using an antibody to CSF-1R, I believe this non-responding population of cells to be contaminating cells, most likely residual monocytes. Alternatively, this small population of non-responding cells may be less differentiated than the majority of the neutrophil cells isolated. Since the kidney is the major hematopoietic organ in fish, akin to that of bone marrow in mammals, one would expect to obtain a mixed population of neutrophils at different differentiation stages using density gradient separation. The differentiation of neutrophils in the kidney from promyelocyte to myelocyte to

metamyelocyte in the kidney and then the appearance of the multi-lobed or segmented nucleus have been documented in a closely related species, the zebrafish [28, 29]. However, to fully address the question of the identity of the non-responding cells one would require specific goldfish granulocyte reagents. For example, granulocyte-specific monoclonal antibodies are available for Atlantic salmon [35] and have been shown to recognize Atlantic cod and trout granulocytes [35-37]. To my knowledge, these types of antibodies have not been developed for goldfish granulocytes. Additionally, isolation and cultivation of progenitor cells from goldfish kidney in the presence of G-CSF may provide a means by which kidney-derived granulocyte cultures could be established similar to the development of macrophages from these precursors in the presence of M-CSF [38].

I also examined goldfish neutrophil response to the pathogen *A. salmonicida*. Neutrophils exhibited a chemotactic response toward live bacteria and bacteria-conditioned supernatants, a degranulation response and an intense respiratory burst response when exposed to live bacteria. These observations corroborate those Lamas and Ellis [24] as well as Jin *et al*. [39] who showed that excretory/secretory products of a related bacterium, *A. hydrophilia*, were highly chemoattractive for neutrophils. This may be a mechanism by which bacteria can overwhelm the fish immune system and evade host defense [40, 41].

Fish neutrophils, unlike that of mammalian neutrophils, have been previously reported to possess a longer half-life and remain functional for extended periods [24]. In accordance with these studies by Lamas and Ellis [24], I also observed neutrophils to possess a sustained capacity to functionally respond as measured through a respiratory burst response to the mitogen PMA.

A similar percentage of cells were observed to respond to PMA activation at 0 and 24 hours post isolation, however, the percentage of cells drastically decreased after 48 hours of cultivation. Based on these data, I believe that my method of goldfish neutrophil isolation and cultivation can be used for functional assessment of neutrophils. However, I have not tested the ability of this isolation system to maintain functional differences between fish exposed to different treatments, such as neutrophil responses from fish that are infected versus non-infected, or fish treated with immuno-regulatory molecules compared to controls. Thus, further experimentation examining the interference of cultivation on neutrophil functions remains to be done.

This is the first study on the isolation and comprehensive functional characterization of goldfish neutrophils. My findings will be useful for further analysis of the mechanisms of inflammation in goldfish and other teleost species and the examination of host response to pathogens.

Table 7.1: Goldfish neutrophils are capable of producing reactive oxygen intermediates after 96 hours in culture. Percentage of goldfish neutrophils that undergo respiratory burst when stimulated for with HBSS, hydrogen peroxide or 0.1 μg/mL, 1 μg/mL or 10 μg/mL of PMA following time of *in vitro* culture.

Time (hrs)	Per cent (%) cells producing reactive oxygen intermediates following treatment				
	HBSS	Hydrogen peroxide	0.1 μg/mL PMA	1 μg/mL PMA	10 μg/mL PMA
0	5.3	88.7	76.2	71.4	75.1
24	4.7	92.7	85.1	77.9	71.5
48	5.2	75.9	63.7	57.2	60.0
72	5.2	57.7	50.4	41.7	51.3
96	5.0	52.7	44.8	45.3	34.7

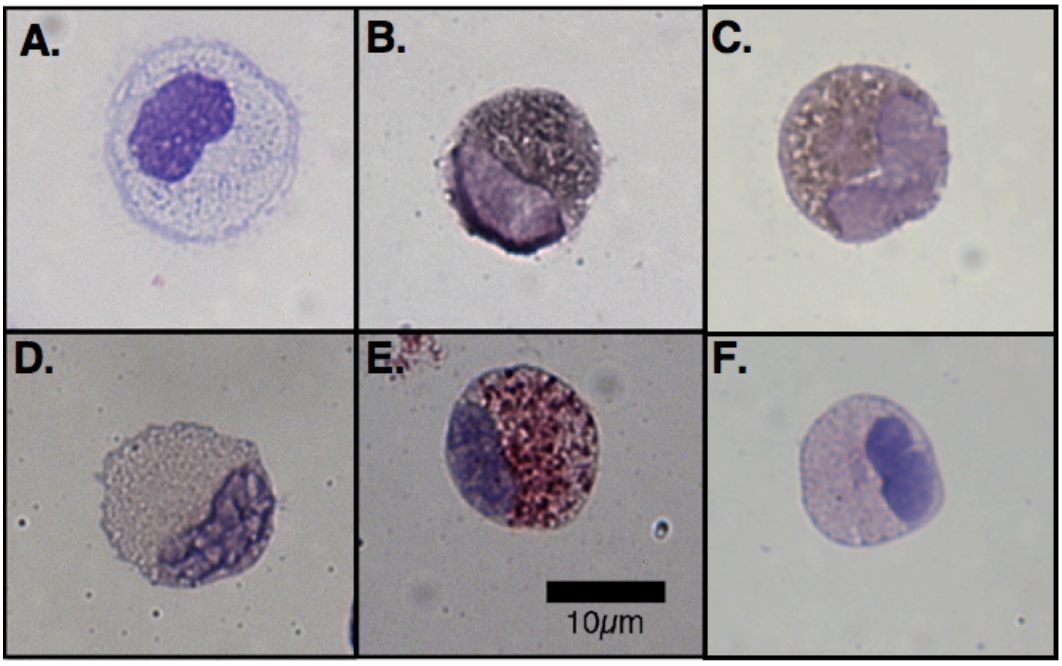


Figure 7.1: Bright field images of goldfish kidney neutrophils.
Cells were stained for Hematoxylin-Eosin (A), Sudan Black B (B), myeloperoxidase (C), non-specific esterase (D), acid-phosphatase (E), and Periodic Acid Schiff (F). Scale =10 μm.

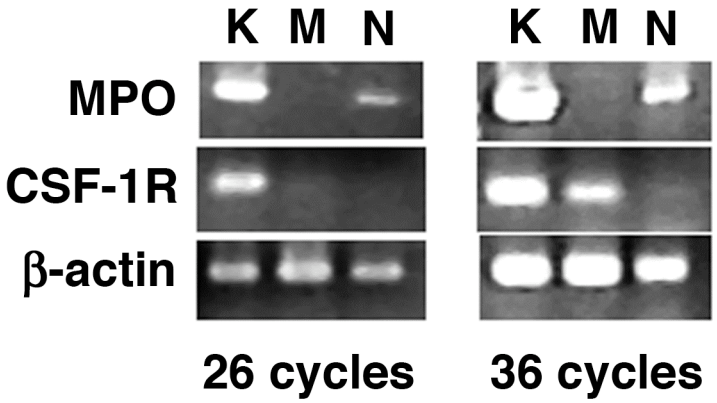


Figure 7.2: Myeloperoxidase and CSF-1R expression in goldfish kidney neutrophils.
RNA was isolated and reverse transcribed into cDNA from goldfish kidney tissue (K), goldfish *in vitro* derived kidney macrophages (M) from day 10 PKM cultures or from overnight cultured goldfish neutrophils (N). The expression of myeloperoxidase (MPO), membrane bound colony-stimulating factor-1 receptor (CSF-1R) and β-actin were examined after 26 and 36 cycles of RT-PCR. Thermocycling parameters were: 2 minutes 94°C, followed by 26 or 36 cycles of 94°C 30s, 60°C (myeloperoxidase) /68°C (CSF-1R) /65°C (β-actin) 30s (respectively), 72°C 1.5 minutes, then an extension at 72°C for 15 minutes. PCR products were visualized on a 1% Tris-acetate-EDTA agarose gel and stained with ethidium bromide.

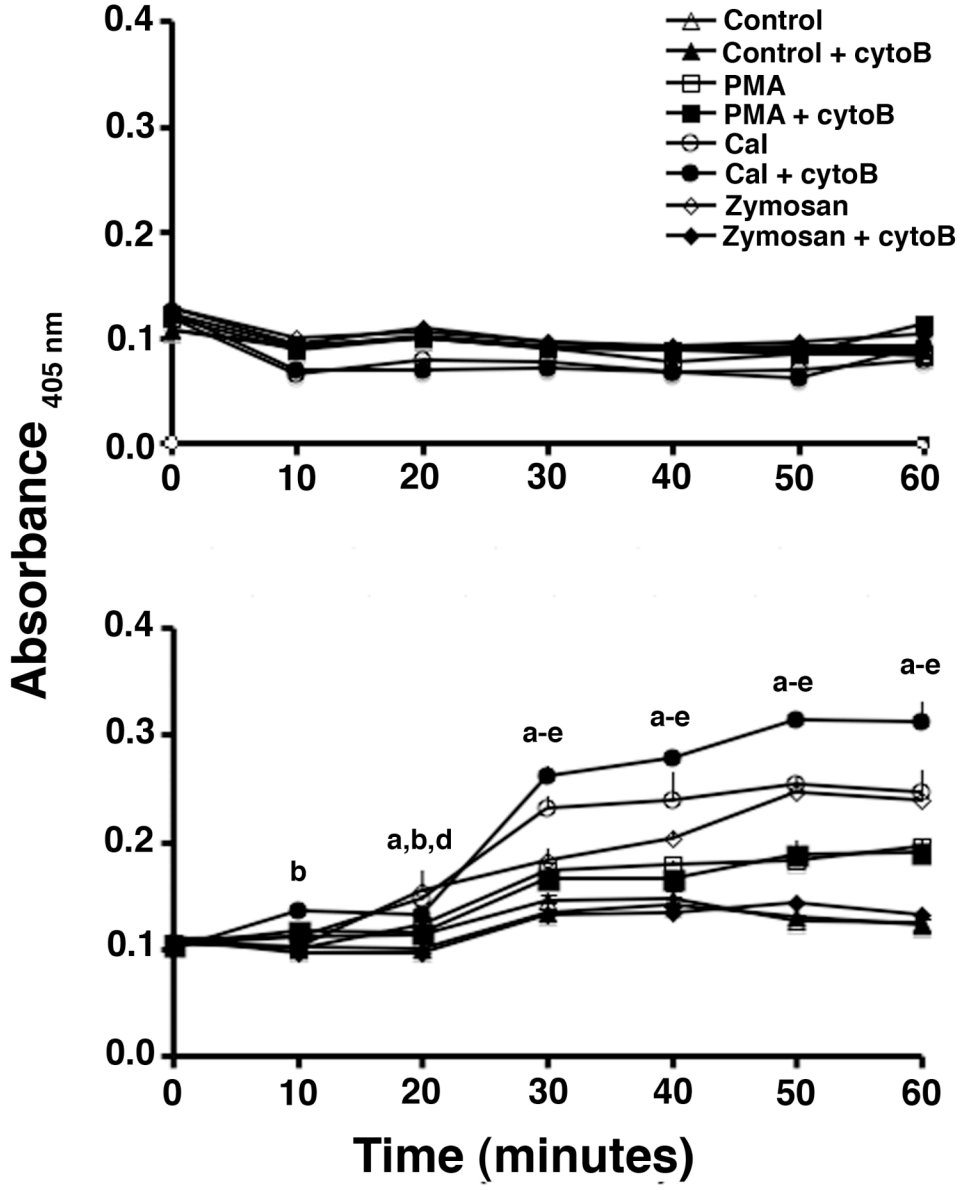


Figure 7.3: Neutrophils degranulate in response to different mitogens in a time dependent manner.
Cells were re-suspended in HBSS without Ca^{2+}, Mg^{2+} (A) or in HBSS with Ca^{++}, Mg^{2+} (B). Treatments consisted of HBSS, 5 μg/mL CaI, 1 μg/mL PMA, or 200 μg/mL Zymosan in the presence or absence of 5μg/mL cytochalasin B (n = 3). The lower case letters represent when treatments were significantly different from the treatment matched control: (a) represents CaI alone, (b) CaI plus cytochalasin B, (c) PMA alone, (d) PMA plus cytochalasin B, (e) Zymosan, (f) Zymosan plus cytochalasin B. ($P < 0.05$) experimental group versus control.

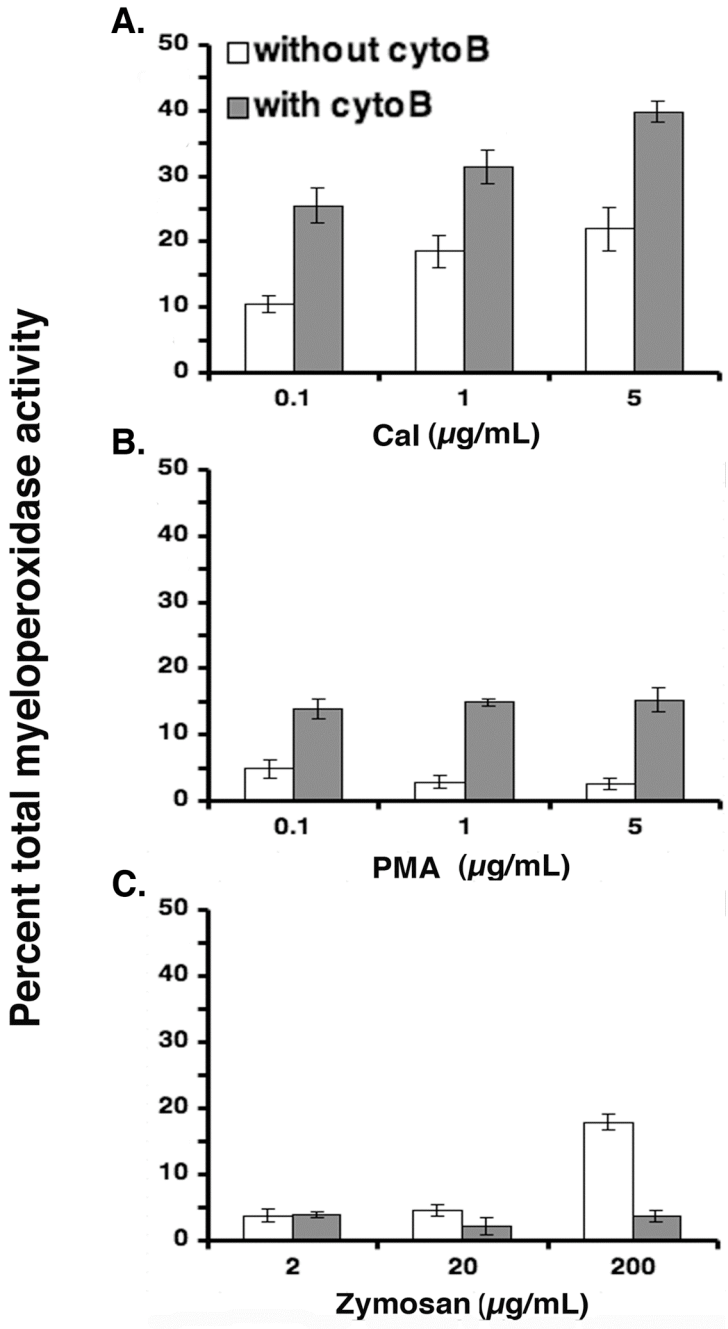


Figure 7.4: Dose dependent degranulation of goldfish neutrophils toward mitogens.
Cells in HBSS with Ca^{2+} and Mg^{2+} were stimulated with different concentrations of (A) CaI, (B) PMA, or (C) Zymosan in the presence or absence of 5μg/mL cytochalasin B for thirty minutes. Cells in either HBSS with or without cytochalasin B were negative controls and were subtracted from experimental and lysed neutrophil absorbencies. Degranulation responses were expressed as percent of total myeloperoxidase from lysed neutrophils (n = 4).

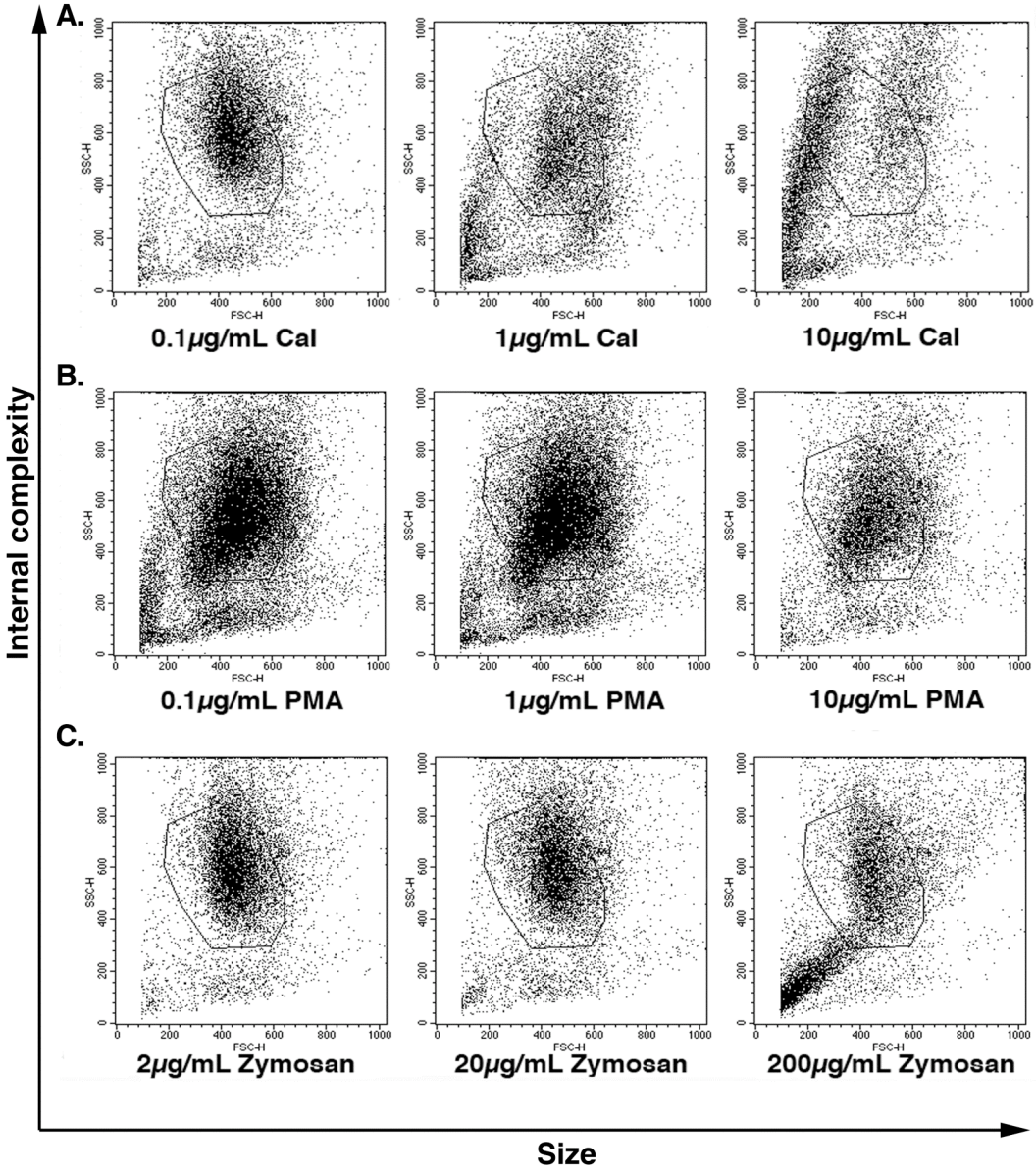


Figure 7.5: Flow cytometry analysis of goldfish neutrophils towards mitogens in a dose dependent manner.
Goldfish neutrophils were analyzed by flow cytometry based on forward scatter (FSC, size) and side scatter (SSC, internal complexity) in response to (A) Calcium ionophore (CaI), (B) PMA, or (C) zymosan at different concentrations in the absence of cytochalasin B after thirty minutes. The data presented are a representative experiment of three independent experiments that were performed.

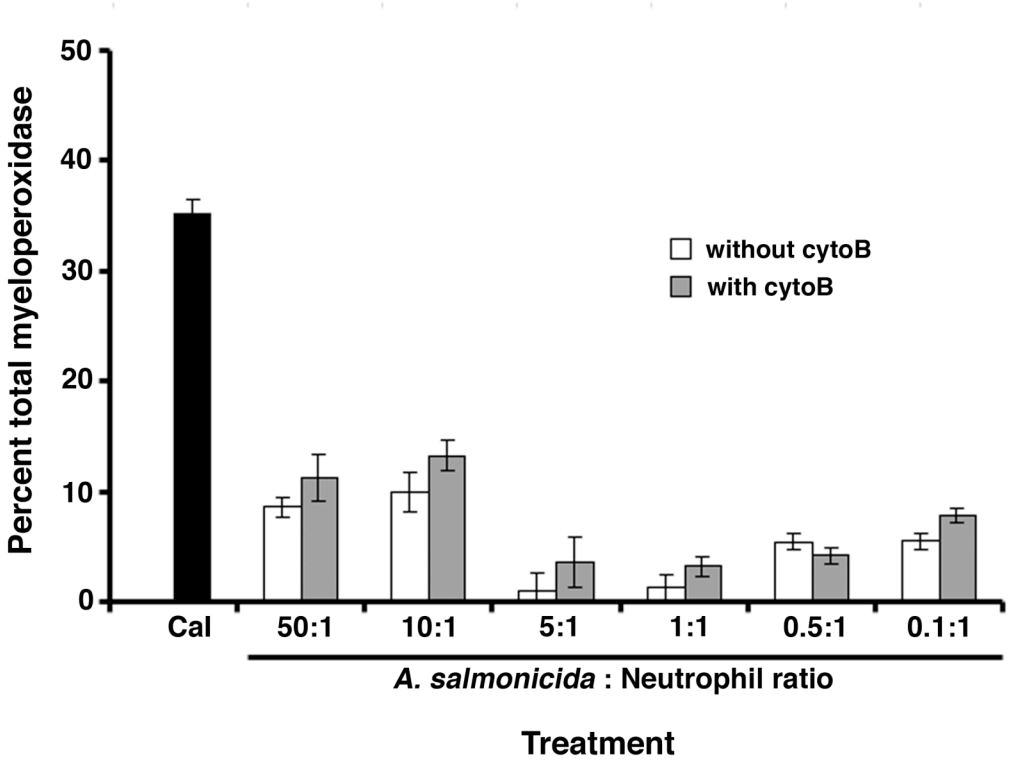


Figure 7.6: *A. salmonicida* A449 induced primary degranulation of neutrophils.
Neutrophils in HBSS with Ca^{2+} and Mg^{2+} were seeded at 50,000 cells per well in triplicate. Live *A. salmonicida* was resuspended in HBSS with calcium and magnesium at concentrations ranging from 2 x 10^5 – 1 x 10^8 bacterial cells/mL, corresponding to *A. salmonicida* to neutrophil ratios of 0.1:1 – 50:1 and added to the wells containing neutrophils in the presence or absence of 5μg/mL cytochalasin B. 5 μg/mL CaI plus cytochalasin B was used as a positive control. Degranulation responses were expressed as percent of total myeloperoxidase from lysed neutrophils (n = 3).

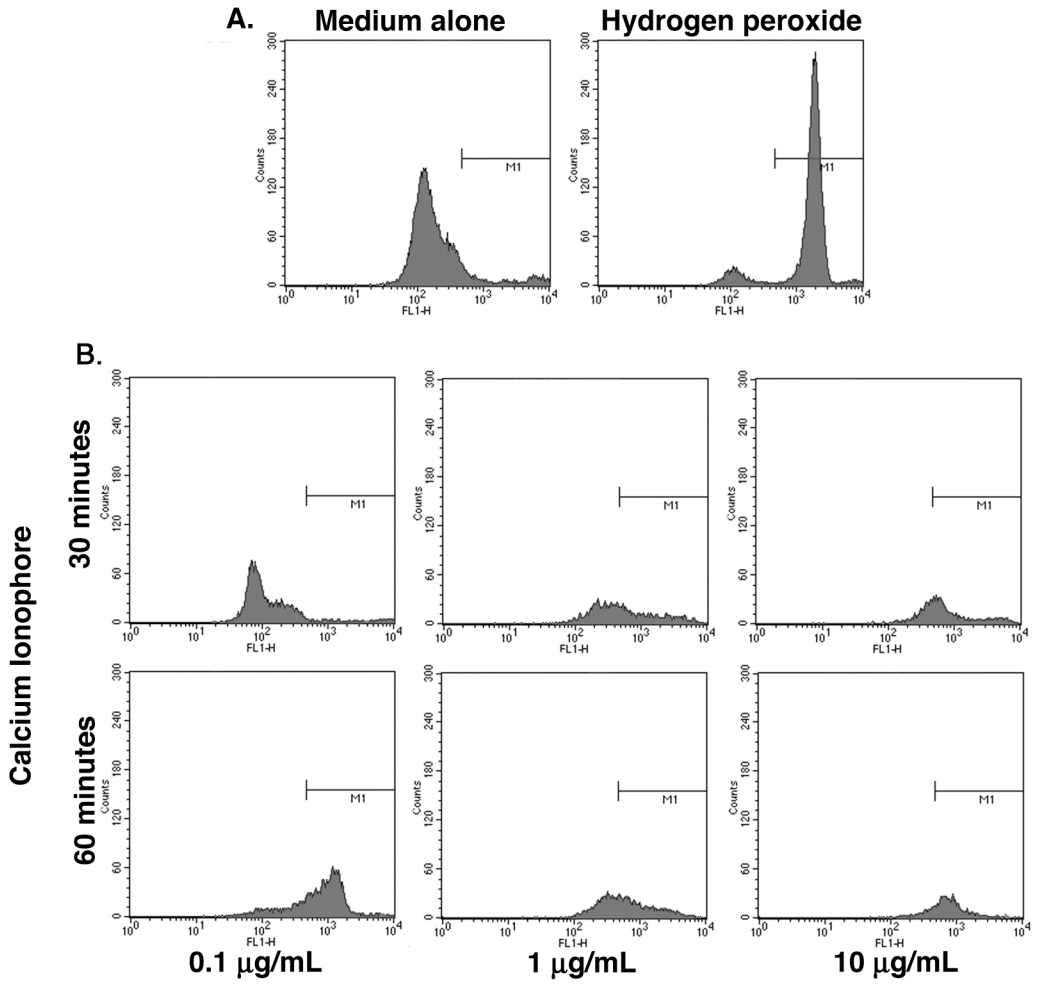


Figure 7.7: Assessment of the production of reactive oxygen intermediates by neutrophils when stimulated with CaI.
Cells in incomplete NMGFL-15 medium were incubated with (A) HBSS without Ca^{++}, Mg^{2+} as a negative control (left panel) or hydrogen peroxide as a positive control (right panel). Neutrophils were treated with (B) 0.1 μg/mL, 1 μg/mL, or 10 μg/mL of CaI, and analyzed by flow cytometry at 30 minutes (top panels) or 60 minutes (bottom panels) post treatment. The data presented are a representative experiment of three that were performed.

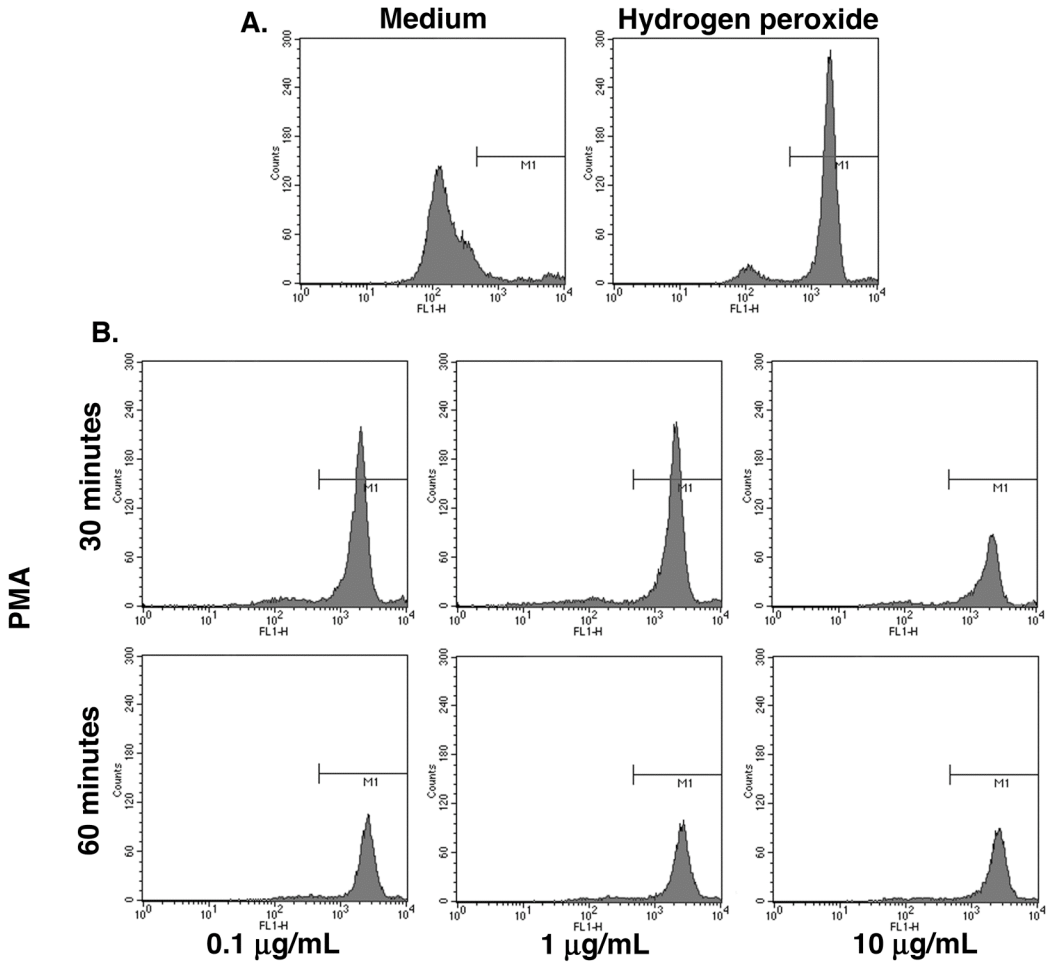


Figure 7.8: Assessment of the production of reactive oxygen intermediates by neutrophils when stimulated with PMA.
Cells in incomplete NMGFL-15 medium were incubated with (A) HBSS without Ca^{++}, Mg^{2+} as a negative control (left panel) or hydrogen peroxide as a positive control (right panel). Neutrophils were treated with (B) 0.1 μg/mL, 1 μg/mL, or 10 μg/mL of PMA, and analyzed by flow cytometry at 30 minutes (top panels) or 60 minutes (bottom panels) post treatment. 10,000 events were analyzed per sample. The data presented are a representative experiment of three that were performed.

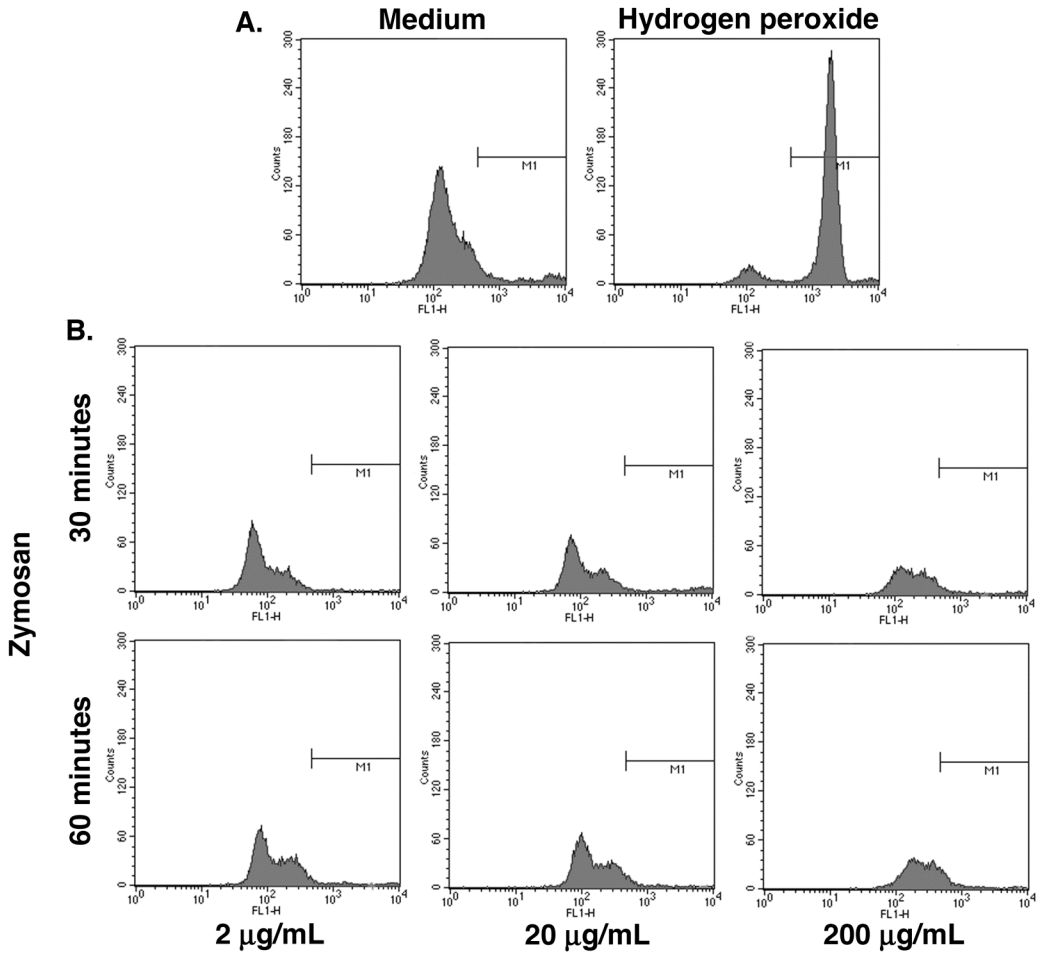


Figure 7.9: Assessment of the production of reactive oxygen intermediates by neutrophils when stimulated with zymosan.
Cells in incomplete NMGFL-15 medium were incubated with (A) HBSS without Ca^{++}, Mg^{2+} as a negative control (left panel) or hydrogen peroxide as a positive control (right panel). Neutrophils were treated with (B) 2 μg/mL, 20 μg/mL, or 200 μg/mL of zymosan, and analyzed by flow cytometry after 30 minutes (top panels) or 60 minutes (bottom panels) of treatment. 10,000 events were analyzed per sample. The data presented are a representative experiment of three that were performed.

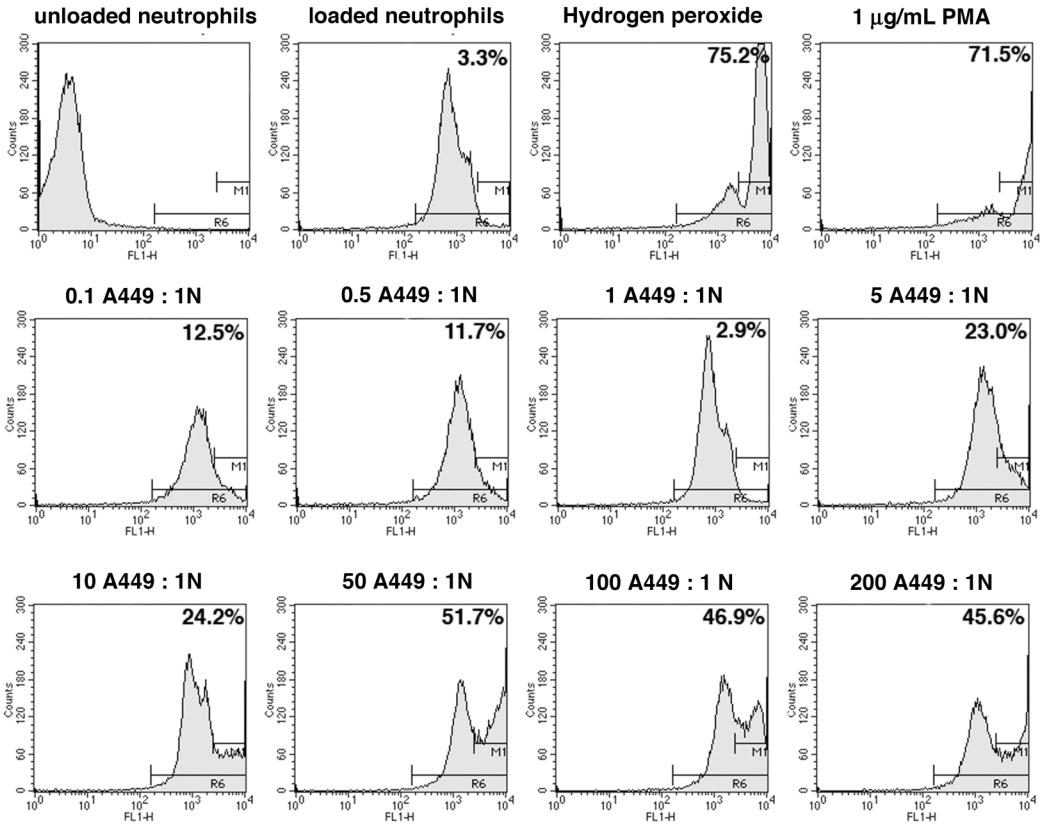


Figure 7.10: *A. salmonicida* A449 induces a respiratory burst response in neutrophils.

Neutrophils were seeded with different concentrations of *Aeromonas* ranging from 0.1-200 bacteria : 1 neutrophil. 1μg/mL PMA was used as a positive control. Following 1 hour stimulation, cells were analyzed using flow cytometry based on number of cells (counts) and fluorescence (FL1-H). Events from the *Aeromonas* were excluded using fluorescence as a discriminator for the gate R6. Per cent indicates cells with a shift in fluorescence to inside the M1 gate, indicative of a respiratory burst response. This is a representative experiment of two independent experiments that were performed.

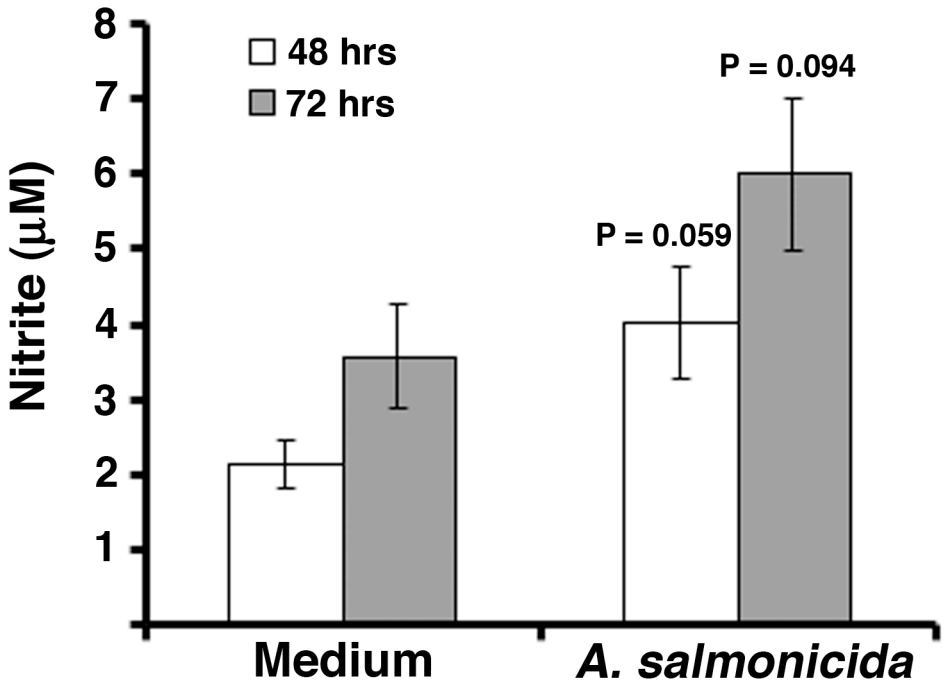


Figure 7.11: Heat-killed *A. salmonicida* A449 induces a small nitric oxide response in neutrophils.
Overnight-cultured neutrophils from individual fish were seeded in duplicate at 100,000 cells per well in complete NMGFL-15 medium. Cells were either treated with complete medium alone (negative control) or a 1:35 dilution of heat-killed *A. salmonicida* A449 in complete medium. Following 48 or 72 hours of incubation at 20°C, nitrite production was indirectly measured using the Griess reaction (n = 4). P-values are indicated compared to the time-matched medium control (student's t-test).

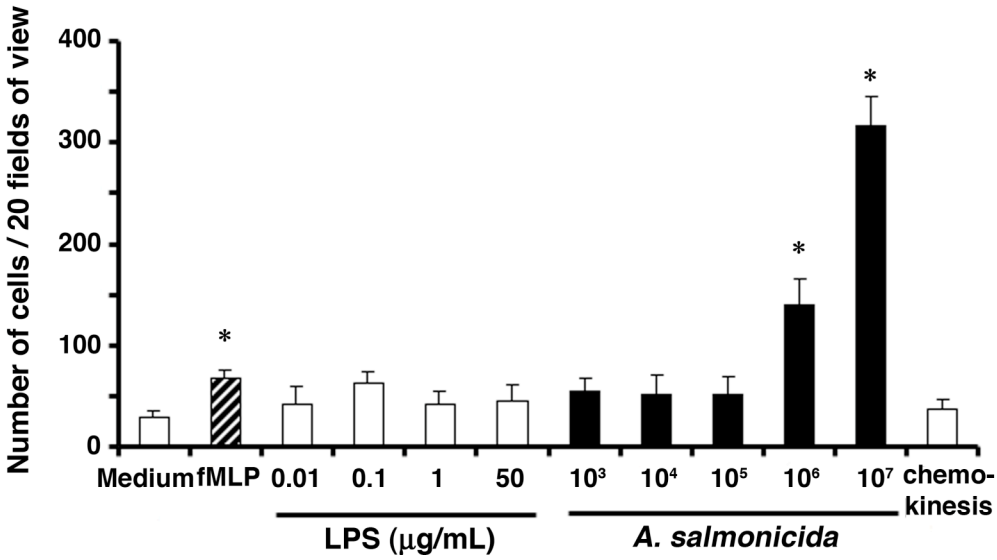


Figure 7.12: Live *Aeromonas salmonicida* A449 induce neutrophil chemotaxis.

Following a one-hour incubation time, duplicate filters were stained with Gills Solution 3 and the total number of cells in 20 random fields of view counted under oil immersion (100X) as a measurement of chemotaxis. The negative control was medium alone, and the positive control was 10 ng/mL fMLP. The chemotactic nature of different concentrations of LPS (w *S. typhimurium*, DIFCO) and live *A. salmonicida* was assessed (n = 4). The chemokinesis control consisted 10^7 *A. salmonicida* A449 in the upper and lower chambers of the chemotaxis wells. A p-value of < 0.05 was considered significant and is denoted by an asterisk.

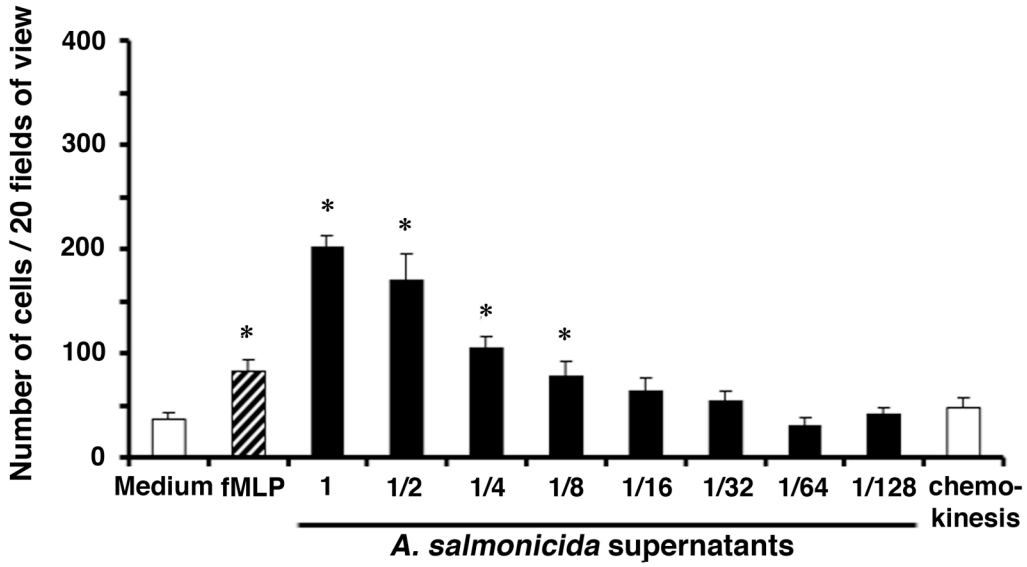


Figure 7.13: *Aeromonas salmonicida* A449 cell-conditioned supernatants are chemoattractive to goldfish kidney neutrophils.
Following a one-hour incubation time, duplicate filters were stained with Gills Solution 3 and the total number of cells in 20 random fields of view counted under oil immersion (100X) as a measurement of chemotaxis. The negative control was medium alone, and the positive control was 10 ng/mL fMLP. The chemotactic nature of different dilutions of *A. salmonicida* was assessed (n = 4). The chemokinesis control consisted of the equivalent of half dilutions the *A. salmonicida* A449 supernatants in the upper and lower chambers of the chemotaxis wells. A p-value of < 0.05 was considered significant and is denoted by an asterisk.

7.4 References:

[1] **Hallett MB, Dewitt S.** Ironing out the wrinkles of neutrophil phagocytosis. Trends Cell Biol. 2007;17(5):209-14.

[2] **Nathan C.** Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006;6(3):173-82.

[3] **Pham CT.** Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol. 2006;6(7):541-50.

[4] **Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al.** Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

[5] **Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al.** Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem. 2004;279(42):44123-32.

[6] **Urban CF, Reichard U, Brinkmann V, Zychlinsky A.** Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol. 2006;8(4):668-76.

[7] **Zinkl JG, Cox WT, Kono CS.** Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish. Comparative Haematology International. 1991;1(4):187-95.

[8] **Palic D, Andreasen CB, Frank DE, Menzel BW, Roth JA.** Gradient separation and cytochemical characterisation of neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Fish Shellfish Immunol. 2005;18(3):263-7.

[9] **Harun NO, Zou J, Zhang YA, Nie P, Secombes CJ.** The biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. Dev Comp Immunol. 2008;32(6):673-81.

[10] **Steinhagen D, Jendrysek S.** Phagocytosis by carp granulocytes: in vivo and *in vitro* observations. Fish Shellfish Immunol. 1994;4521-5.

[11] **Mayumi M, Takeda Y, Hoshiko M, Serada K, Murata M, Moritomo T, et al.** Characterization of teleost phagocyte NADPH oxidase: molecular cloning and expression analysis of carp (*Cyprinus carpio*) phagocyte NADPH oxidase. Mol Immunol. 2008;45(6):1720-31.

[12] **Palic D, Andreasen CB, Menzel BW, Roth JA.** A rapid, direct assay to measure degranulation of primary granules in neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Fish Shellfish Immunol. 2005;19(3):217-27.

[13] **Palic D, Ostojic J, Andreasen CB, Roth JA.** Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol. 2007;31(8):805-16.

[14] **Palic D, Andreasen CB, Ostojic J, Tell RM, Roth JA.** Zebrafish (Danio rerio) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. J Immunol Methods. 2007;319(1-2):87-97.

[15] **Kurata O, Okamoto N, Ikeda Y.** Neutrophilic granulocytes in carp, *Cyprinus carpio*, possess a spontaneous cytotoxic activity. Dev Comp Immunol. 1995;19(4):315-25.

[16] **Palic D, Andreasen CB, Herolt DM, Menzel BW, Roth JA.** Immunomodulatory effects of beta-glucan on neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820). Dev Comp Immunol. 2006;30(9):817-30.

[17] **Kampen AH, Tollersrud T, Larsen S, Roth JA, Frank DE, Lund A.** Repeatability of flow cytometric and classical measurement of phagocytosis and respiratory burst in bovine polymorphonuclear leukocytes. Vet Immunol Immunopathol. 2004;97(1-2):105-14.

[18] **Quade MJ, Roth JA.** A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Immunol Immunopathol. 1997;58(3-4):239-48.

[19] **Graham S, Jeffries AH, Secombes CJ.** A novel assay to detect macrophage bactericidal activity in fish: factors influencing the killing of *Aeromonas salmonicida*. Journal of Fish Diseases. 1998;11389-96.

[20] **Dror M, Sinyakov MS, Okun E, Dym M, Sredni B, Avtalion RR.** Experimental handling stress as infection-facilitating factor for the goldfish ulcerative disease. Vet Immunol Immunopathol. 2006;109(3-4):279-87.

[21] **Wiklund T, Dalsgaard I.** Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. Dis Aquat Organ. 1998;32(1):49-69.

[22] **Olivier G, Moore AR, Fildes J.** Toxicity of *Aeromonas salmonicida* cells to Atlantic salmon Salmo salar peritoneal macrophages. Dev Comp Immunol. 1992;16(1):49-61.

[23] **Daly JG, Kew AK, Moore AR, Olivier G.** The cell surface of *Aeromonas salmonicida* determines in vitro survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. Microb Pathog. 1996;21(6):447-61.

[24] **Lamas J, Ellis AE.** Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immunol. 1994;4201-19.

[25] **Lamas J, Ellis AE.** Electron microscopic observations of the phagocytosis and subsequent fate of *Aeromonas salmonicida* by Atlantic salmon neutrophils in vitro. Fish Shellfish Immunol. 1994;4539-46.

[26] **Kemenade B, Groeneveld A, Rens B, Rombout J.** Characterization of Macrophages and Neutrophilic Granulocytes from the Pronephros of Carp (*Cyprinus Carpio*). J Exp Biol. 1994;187(1):143-58.

[27] **Kemenade B, Daly JG, Groeneveld A, Wiegertjes GF.** Multiple regulation of carp (*Cyprinus carpio* L.) macrophages and neutrophilic granulocytes by

serum factors: influence of infection with atypical *Aeromonas salmonicida*. Vet Immunol Immunopathol. 1996;51189-200.

[28] **Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE.** Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood. 2001;98(10):3087-96.

[29] **Crowhurst MO, Layton JE, Lieschke GJ.** Developmental biology of zebrafish myeloid cells. Int J Dev Biol. 2002;46(4):483-92.

[30] **Barreda DR, Hanington PC, Stafford JL, Belosevic M.** A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[31] **Castro R, Piazzon MC, Noya M, Leiro JM, Lamas J.** Isolation and molecular cloning of a fish myeloperoxidase. Mol Immunol. 2008;45(2):428-37.

[32] **Barker D, Holliday J.** Blood lymphocyte and neutrophil response of cultured rainbow trout, *Oncorhynchus mykiss*, administered varying dosages of an oral immunomodulator-'Fin-ImmuneTM'. World Academy of Science, Engineering and Technology. 2009;499-13.

[33] **Serada K, Moritomo T, Teshirogi K, Itou T, Shibashi T, Inoue Y, et al.** Comparison of respiratory burst activity of inflammatory neutrophils in ayu (*Plecoglossus altivelis*) and carp (*Cyprinus carpio*). Fish Shellfish Immunol. 2005;19(4):363-73.

[34] **Zurier RB, Hoffstein S, Weissmann G.** Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. Proc Natl Acad Sci U S A. 1973;70(3):844-8.

[35] **Hamdani SH, McMillan DN, Pettersen EF, Wergeland H, Endresen C, Ellis AE, et al.** Isolation of rainbow trout neutrophils with an anti-granulocyte monoclonal antibody. Vet Immunol Immunopathol. 1998;63(4):369-80.

[36] **Pettersen EF, Fyllingen I, Kavlie A, Maaseide NP, Glette J, Endresen C, et al.** Monoclonal antibodies reactive with serum IgM and leukocytes from Atlantic salmon (*Salmo salar* L.) Fish Shellfish Immunol. 1995;5275-87.

[37] **Ronneseth A, Wergeland HI, Pettersen EF.** Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2007;23(3):493-503.

[38] **Hanington PC, Wang T, Secombes CJ, Belosevic M.** Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[39] **Jin GF, Chopra AK, Houston CW.** Stimulation of neutrophil leukocyte chemotaxis by a cloned cytolytic enterotoxin of Aeromonas hydrophila. FEMS Microbiol Lett. 1992;77(1-3):285-9.

[40] **Mutoloki S, Brudeseth B, Reite OB, Evensen O.** The contribution of Aeromonas salmonicida extracellular products to the induction of inflammation in Atlantic salmon (*Salmo salar* L.) following vaccination with oil-based vaccines. Fish Shellfish Immunol. 2006;201-11.

[41] **Rodriguez I, Novoa B, Figueras A.** Immune response of zebrafish (*Danio rerio*) against a newly isolated bacterial pathogen Aeromonas hydrophila. Fish Shellfish Immunol. 2008;25(3):239-49.

Chapter 8: Characterization of granulocyte colony-stimulating factor receptor of the goldfish[5]

8.1 Introduction

Neutrophils are one of the first cells at the site of infection or tissue injury and possess an arsenal of potent antimicrobial responses for the elimination of infectious agents such as phagocytosis, respiratory burst, degranulation and release of toxic compounds and the ability to send out neutrophil extracellular traps (NETs) [1-5]. The potential toxic threat that neutrophils pose to host tissue should they undergo a spontaneous response is limited due to the short lifespan of neutrophils *in vivo* and *in vitro* (~90 hrs and ~8 hrs, respectively) controlled by pre-programmed apoptosis [6]. Thus, the continuous production of neutrophils, and the necessity of the immune system to respond to pathogens by increasing neutrophil numbers, must be tightly regulated. It is primarily through the action of granulocyte colony-stimulating factor (GCSF) that signals through its receptor (GCSFR) that is required for the development of neutrophils from bone marrow progenitors to committed neutrophil precursors (reviewed in [7]).

GCSFR is a member of the class I cytokine receptor superfamily which includes other receptors such as GP130. The structure of GCSFR is comprised of a signal peptide, an immunoglobulin-like domain, a cytokine receptor homology (CRH) domain, containing the class I cytokine receptor superfamily motif, W-S-X-W-S, three fibronectin domains, a transmembrane domain, and an

[5] A version of this chapter has been published: Katzenback and Belosevic, 2012. *Developmental and Comparative Immunology,* 36: 199-207.

intracellular cytoplasmic signaling domain containing three motifs termed Box 1, Box 2, and Box 3, important for signal transduction [7]. Binding of a homodimeric GCSF complex to the Ig and CRH domains of two GCSF receptors triggers intracellular signaling through the JAK/STAT, Ras/Raf/Erk, or PI3-kinase pathways [8-10]. These signaling pathways ultimately lead to the migration, survival, proliferation, and differentiation of neutrophils. While there are reports of GCSFR expression on other hematopoietic cells such as monocytes [11] and lymphocytes, as well as some non-hematopoietic cells, GCSFR is primarily expressed on neutrophils and their precursors [7, 8].

Previously, I identified and characterized goldfish neutrophil cells that are cytochemically and functionally similar to other vertebrate neutrophils [12]. While a number of studies have examined the antimicrobial arsenal of teleost neutrophils such as chemotaxis, phagocytosis, respiratory burst, degranulation and NETs [13-18], the process of neutrophil development, including the receptors and growth factors that are involved, have remain largely unknown in teleosts. To my knowledge, there is only one study that directly examined GCSFR in zebrafish myeloid cell development. Liongue et al. (2009a) [7, 8], demonstrated the presence of *gcsf* and *gcsfr* mRNA in zebrafish, and showed that zebrafish injected with *gcsfr* morpholinos had reduced numbers of myeloid cells as well as neutropenia. Interestingly, neutrophils were still present in morpholino-injected fish, albeit at low numbers, presumably due to a GCSF/GCSFR-independent developmental pathway [19].

In this chapter, I describe the identification and *in silico* and molecular analysis of a goldfish *gcsfr*. Specifically, I show that goldfish kidney neutrophils have high mRNA levels of *gcsfr*, and that the mRNA levels of *gcsfr* are

modulated in response to neutrophil activation. The results suggest that GCSFR may be involved in the development and activation of goldfish neutrophils. The objectives of the experiments described in this chapter were to (1) identify the goldfish *gcsfr* transcript (2) determine the gene copy number of *gcsfr,* (3) examine the mRNA levels of *gcsfr* in tissues and in progenitor cells, kidney neutrophils, and populations of neutrophils from the blood and spleen, (4) examine the transcription factor mRNA levels in purified kidney neutrophils, and lastly (5) assess the *gcsfr* mRNA levels in kidney neutrophils in response to mitogens and to heat-killed *A. salmonicida*.

8.2 Results

8.2.1 Phylogenetic analysis of goldfish GCSFR

Phylogenetic analysis of the goldfish GCSFR predicted protein sequence placed it with other teleost GCSFR sequences and showed the most similarity to *Danio rerio* GCSFR (Accession number NM_001113377) with 56% identity (Fig. 8.1). The protein is predicted to contain many of the conserved domains found within the GCSFR members of the class I cytokine receptor family. These domains include a signal peptide sequence predicted to be cleaved between amino acid (AA) 26 and 27, an Ig-like domain with four cysteine residues present required for disulphide bonding and structure, and a domain containing cysteine residues and a signature motif W-S-X-W-S characteristic of the class I cytokine receptor homology (CRH) domain (Fig. 8.2). Additionally, the goldfish GCSFR protein was predicted to possess three fibronectin domains, a transmembrane domain, as well as a cytoplasmic domain (Fig. 8.2). Within the cytoplasmic

domain, there were various motifs identified that are involved in intracellular signaling. These include 6 tyrosine residues, and a strong conservation of sequence in the Box 1 and 3 regions, with particular conservation of the STQPLL motif important for receptor internalization in Box 3 (Fig. 8.2). The 3' untranslated region (UTR) possessed two instability motifs with the sequence AUUUA, an AATAAA polyadenylation signal, as determined by the PolyApred program [20], and a poly A tail.

8.2.2 Southern blot

A Southern blot on goldfish genomic DNA was performed to detect a potential gene duplication. Genomic DNA isolated from goldfish blood was digested with the restriction enzymes KpnI, HindIII, XbaI, PstI, PaeI, and TaiI overnight. The restriction enzymes were chosen based on their restriction cut sites. KpnI, HindIII, XbaI, PstI, and PaeI did not cut *gcsfr* genomic DNA in the exon that was chosen as the probe. Only TaiI was predicted to cut within the exon probe. The digested goldfish genomic DNA was run on an agarose gel for 11 hrs, and transferred to a nylon membrane. The membrane was blocked and incubated with a probe that recognized the predicted second exon of goldfish *gcsfr* that encodes for the Ig-like domain in the GCSFR protein. The blot was probed and washed at 60^{o}C. The detection reagent was added to the blot to produce a chemilluminescent signal, and the membrane was exposed to film for 1 hour or 2 hours (Fig. 8.3, left and right panels, respectively).

As expected, two bands were observed in the TaiI genomic DNA digested lane (Fig. 8.3). In the lanes in which the genomic DNA was digested with KpnI,

HindIII, and XbaI, two bands were observed. In the lanes in which the genomic DNA was digested with PstI or PaeI, three bands were observed (Fig. 8.3). This may suggest the presence of a second *gcsfr* gene, or this may represent additional alleles of the same gene due to the tetraploid nature of the goldfish. It should be noted, however, that the additional bands that are detected are fainter than the first band, suggesting that this probe is not binding as strongly to this genomic DNA fragment (Fig. 8.3). Therefore, I cannot rule out the possibility that these additional bands might represent another member of the class I cytokine receptor superfamily that has yet to be identified in goldfish.

8.2.3 Examination of *gcsfr* mRNA levels in goldfish tissues and cell populations

The blood, gill, brain, heart, spleen, kidney and intestine were harvested from four fish and the mRNA levels of goldfish *gcsfr* were assessed in these tissues using quantitative PCR (qPCR). The *gcsfr* mRNA levels were highest in kidney and was ~ 15 fold higher in the kidney compared to blood (Fig. 8.4). The *gcsfr* mRNA levels were significantly higher in the spleen, gill, and brain, while *gcsfr* mRNA levels in the heart and intestine were not significantly different compared to the *gcsfr* mRNA level in the blood (Fig. 8.4).

I have previously isolated and characterized neutrophils from the goldfish kidney with > 92% purity [12], and wanted to assess whether these goldfish neutrophils, like mammalian neutrophils, expressed *gcsfr*. Neutrophils were isolated from four individual fish and cultured overnight prior to RNA isolation. I then compared the levels of *gcsfr* mRNA in the purified neutrophils to that of the

kidney tissue isolated from four animals using qPCR. The neutrophils had significantly higher *gcsfr* mRNA levels (~10 fold) compared to the kidney tissue (Fig. 8.5A). These results suggest the presence of a large population of neutrophils within the goldfish kidney, which accounts for the high levels of *gcsfr* mRNA observed in this tissue.

It is known that GCSF receptors increase in number with development of committed neutrophil progenitor cells into mature neutrophils [21-23]. To determine whether a similar pattern existed in goldfish, *gcsfr* mRNA levels were assessed in goldfish kidney progenitor cells (sorted R1 cells from PKM cultures), neutrophils, and in neutrophil-enriched cell populations from peripheral blood leukocytes (PBLs) and splenocytes. Three to four fish were used for the isolation of each cell population. The mRNA levels of *gcsfr* were ~20 fold higher in the purified kidney neutrophils compared to the *gcsfr* mRNA levels in the goldfish progenitor cells (Fig. 8.5B). However, due to the difficulty of purifying neutrophils from goldfish blood and spleen, I only achieved ~13% purity of neutrophils from the PBLs, and ~4% from the splenocytes as previously described in chapter 7, I did not observe high levels of *gcsfr* mRNA in these cell populations because *gcsfr* mRNA was most likely diluted out by the large number of contaminating cells present (Fig. 8.5B). However, there were significantly higher mRNA levels of *gcsfr* in the neutrophil enriched peripheral blood leukocytes compared to that of progenitor cells (Fig. 8.5B).

8.2.4 Comparison of the expressions of hematopoietic transcription factors in goldfish neutrophils and progenitors.

I next examined the transcription factors (TFs) involved in the development of neutrophils from progenitor cells. The mRNA levels of the transcription factors *runx1*, *cmyb*, *gata2*, *pu.1*, *mafb*, *cjun*, *egr1*, *cebpα*, *gata1*, *lmo2*, *gata3* and *pax5* were analyzed in progenitor cells and neutrophils isolated from four individual fish by quantitative PCR. The TFs were grouped into four categories based on their major involvement in lineage decisions [24]. The TFs *runx1*, *cmyb* and *gata2* were used as markers of early progenitor cells. Neutrophils had significantly lower mRNA levels of *runx1* ($P < 0.05$) and *cmyb* ($P < 0.08$) compared to progenitor cells (Fig. 8.6A). The mRNA levels of *gata2* were not significantly different between neutrophils and progenitor cells (Fig. 8.6A).

The TFs in the second group, *pu.1*, *mafb*, *cjun*, *egr1*, and *cebpα* were chosen based on their involvement in the macrophage/granulocyte lineages. Neutrophils had significantly higher mRNA levels of *pu.1* and *cebpα*, while *cjun* and *egr1* mRNA levels were significantly lower, compared to the mRNA levels of these TFs in the progenitor cells (Fig. 8.6B). However, the mRNA levels of *mafb* did not significantly differ between neutrophils and progenitor cells (Fig. 8.6B).

The third group of transcription factors consisting of *gata1* and *lmo2* was selected based on their involvement in the erythroid/megakaryocyte lineages. The neutrophils had significantly lower mRNA levels of *gata1* ($P < 0.05$) and *lmo2* ($P < 0.08$) compared to the mRNA levels of these transcription factors in the progenitor cells (Fig. 8.6C).

Lastly, TFs were chosen based on their involvement in lymphopoiesis; *gata3* as a marker of T-cell development and *pax5* as a master regulator of B-cell

development. Both *gata3* and *pax5* mRNA levels were significantly lower in neutrophils compared to that of the progenitor cells, $P < 0.05$ and $P < 0.08$, respectively (Fig. 8.6D).

8.2.5 Assesment of mRNA levels of *gcsfr* in PKMs and in progenitor cells isolated from PKM cultures

The Belosevic laboratory has previously developed an *in vitro* derived primary kidney macrophage (PKM) culture system in which isolated kidney progenitor cells give rise to monocytes and macrophages with time of culture [25, 26]. To assess the mRNA levels of *gcsfr* in these PKM cells, qPCR was performed on PKM cells from cultures established from individual fish ($n = 3$) after 0, 2, 4 or 6 days of culture. The mRNA levels of *gcsfr* were significantly down-regulated in PKMs at 2 and 4 days of culture ($P < 0.05$), and after 6 days of culture ($P < 0.08$) compared to *gcsfr* mRNA levels in freshly isolated day 0 PKMs (Fig. 8.7A). Next, progenitor cells were sorted from PKMs after 0, 2, and 6 days of cultivation. Four individual PKM cultures were set up for each time point, and progenitor cells sorted. However, there were no significant changes in *gcsfr* mRNA levels in progenitor cells during cultivation (Fig. 8.7B), suggesting that the decrease in *gcsfr* mRNA observed in the PKM cells was due to an increase in monocytes and macrophages and a decrease in the number of progenitor cells in the PKM cultures.

8.2.6 Effects of mitogen treatment on *gcsfr*, *kita*, and *prominin* mRNA levels in neutrophils

Goldfish kidney neutrophils from 5-6 fish, in two independent experiments, were treated with either medium (negative control) or a cocktail of mitogens to examine whether mRNA levels of *gcsfr* could be modulated when neutrophils were activated. Following addition of mitogens, the neutrophils flattened out and became adherent to the bottom of the wells, while neutrophils in medium alone remained in suspension and their morphology did not change throughout the time course of the experiment. Neutrophils were isolated at 1, 3 and 6 hrs post treatment, and the mRNA levels of *gcsfr*, *kita*, and *prominin* examined. The mRNA levels of *gcsfr* were significantly increased in neutrophils treated with mitogens after 1 hr of treatment (5 of the 6 data points were at, or above, the mean of the medium control treatment, $P < 0.05$), and in mitogen-treated neutrophils compared to the time-matched neutrophils treated with medium alone at 3 hrs post treatment (4 of the 5 data points were at or above the mean of the medium control treatment, $P < 0.08$) (Fig. 8.8A). By 6 hrs post treatment, the *gcsfr* mRNA levels in mitogen-treated neutrophils were not significantly different from medium controls (Fig. 8.8A).

I also examined *kita* and *prominin* expressions in neutrophils treated with mitogens. There was a generalized decrease of *kita* expression in mitogen-treated neutrophils at all time points, and the mRNA levels were significantly down-regulated at 3 and 6 hrs post mitogen treatment compared to medium controls (Fig. 8.8B). However, *prominin* mRNA levels were not significantly different between medium-treated and mitogen-treated neutrophils at all time points (Fig. 8.8C).

8.2.7 Effects of heat-killed *A. salmonicida* A449 treatment on *gcsfr*, *kita*, and *prominin* mRNA levels in neutrophils

I next examined whether treating neutrophils with heat-killed *A. salmonicida* A449 would affect *gcsfr*, *kita* and *prominin* mRNA levels after 3 hrs of incubation. Neutrophils from a total of five fish, combined from two replicate experiments, were cultured overnight prior to the experiment. Suspension cells were harvested, seeded at two million cells per well, and treated with medium alone as a negative control or with heat-killed *A. salmonicida* for three hours prior to RNA isolation. There was a significant upregulation of *gcsfr* and *kita* mRNA levels in neutrophils treated with heat-killed *A. salmonicida* A449, compared to the *gcsfr* mRNA levels in medium-treated control neutrophils (Fig. 8.9A). Although the mRNA levels of *gcsfr* and *kita* were variable in kidney neutrophils isolated from individual fish, upon stimulation with *A. salmonicida* A449 the mRNA levels of *gcsfr* and *kita* increased in each case as shown by the corresponding data points connected by lines (Fig. 8.9B and C). The mRNA levels of *prominin* were up-regulated in neutrophils following treatment with heat-killed *A. salmonicida A449* by approximately 1.5 fold compared to that of the medium treated control neutrophils, $P < 0.08$, (Fig. 8.9A and D).

8.3 Discussion

Neutrophils are key players in an inflammatory response and the regulation of their development through GCSFR signaling is important in maintaining neutrophil numbers during homeostatic and emergency conditions. Despite the important role neutrophils play during an innate immune response,

teleost neutrophils and the receptors and ligands that govern their development have remained largely unexplored. Therefore, my aim was to identify the granulocyte colony-stimulating factor receptor of goldfish and examine the expression of this receptor in tissues, cell populations and how the expression of *gcsfr* mRNA is modulated by mitogens and pathogens. The results of these experiments were described in this chapter.

The identified goldfish GCSFR has 823 amino acids and is predicted to have the Ig-like domain and the cytokine receptor homology domain containing the WSXWS motif and cysteine residues, known to be important in binding the homodimeric GCSF ligand in mammalian systems [27, 28], and three fibronectin domains. Additionally, goldfish GCSFR, like the zebrafish GCSFR, appears to have many of the motifs required for intracellular signaling such as the box1, 2, and 3 motifs, and a number of tyrosine residues that are important in JAK/STAT, PI3K, and Ras/Raf/ERK signaling pathways [8, 19]. This suggests that the signaling pathways of GCSFR may be conserved in vertebrates. However, the goldfish GCSFR protein sequence was only 56% identical to the zebrafish GCSFR, which provides further evidence for the selective pressure on the GCSF receptor and ligand pair as previously suggested [29].

Due to the tetraploid nature of goldfish, I performed a Southern blot using a probe directed to the predicted second exon of the identified goldfish GCSFR that composes the Ig-like domain of the predicted GCSFR protein structure. In all restriction enzyme digestions, I observed the presence of more than one band. This suggests that there is most likely another GCSFR gene present in goldfish. Although the additional bands could represent alleles of the same GCSFR gene that I have identified, or quite possibly, represent another member of the class I

cytokine receptor superfamily in goldfish. Many studies on a number of different genes in goldfish have revealed gene duplications and alleles of a particular gene [30-32]. If a second GCSFR gene exists, it would be interesting to determine the sequence and function of this gene. However, this would be a huge undertaking as the goldfish lacks much of the genomic resources available for zebrafish.

To provide insight into the role of the identified GCSFR in teleosts, I examined the distribution of *gcsfr* mRNA in goldfish tissues. Not surprisingly, I observed the greatest level of *gcsfr* mRNA in the kidney and spleen tissues, the major hematopoietic tissues of teleosts. I also observed significantly higher mRNA levels of *gcsfr* in gill and brain tissues. The presence of *gcsfr* in the gill may represent a population of neutrophils that is attracted to the site, as a mechanism to deal with potential pathogens that enter at the gill. Additionally, it has been reported in the mammalian system that GCSFR is expressed in adult neural stem cells of the brain as well as a being broadly expressed in many regions of the brain [33]. Furthermore, treatment of different mice models with GCSF have demonstrated increased angiogenesis, neurogenesis, dendritogenesis, and decreased apoptosis in the brain [33]. While it is clear that GCSF/GCSFR have functions outside of the hematopoietic system in mammals, it is interesting to note that authors did not describe any neuronal defects in *gcsfr* morpholino-injected zebrafish, while they did find defects within the myeloid developmental pathway [19]. Due to the increased levels of *gcsfr* mRNA in the brain tissue of goldfish, it may be interesting to investigate the role of the GCSFR in zebrafish brain development.

The Belosevic laboratory is interested in defining the mechanisms of myelopoiesis in teleosts and I have recently identified and characterized

neutrophils from the goldfish kidney, described in chapter 7 [12]. These neutrophils exhibited cytochemical characteristics of mammalian neutrophils, expressed message for myeloperoxidase but not for the colony stimulating factor -1 receptor (CSF-1R), and were functionally capable of antimicrobial processes such as chemotaxis, degranulation, and respiratory burst towards mitogens and pathogens or their products. However, these kidney neutrophils did not possess the typical multi-lobed or segmented nuclei of a mature mammalian neutrophil [12], and instead appeared to have a kidney-shaped nucleus reminiscent of a myelocyte or metamyelocyte [34].

I have recently identified a number of transcription factors in the goldfish that are known to be involved in hematopoiesis [24] (this topic was described in Chapter 6), and decided to examine the transcription factor profile of the neutrophils compared to progenitor cells to provide further evidence of the identity of these cells. The neutrophils showed a significant increase in *pu.1* and *cebp*α mRNA levels over progenitor cells, important transcription factors in neutrophil development [35], and lower levels of many of the transcription factors involved in the survival and maintenance of early progenitor cells [36, 37], and those transcription factors involved in lymphoid [36, 38] and erythrocyte/megakaryocyte [39, 40] developmental pathways. Furthermore, the mRNA levels of *cjun* and *egr1*, transcription factors involved in monocyte/macrophage development [41, 42], were significantly lower in neutrophils compared to progenitor cells. While these data supported the identity of these cells as neutrophils, I decided to examine if these cells also expressed *gcsfr*. Indeed, purified neutrophils from the goldfish kidney enriched for the level of *gcsfr* mRNA by approximately 10 fold, further supporting that these cells

belong to the neutrophil lineage using *gcsfr* mRNA expression as a marker. The transcription factor results and the expression of *gcsfr* mRNA in the neutrophils, support the presence of activating *pu.1* and *cebpa* transcription factor binding sites upstream of zebrafish and mammalian GCSFR genes [19, 43].

It has been reported that the expression of GCSFR increases in committed neutrophil progenitor cells as they mature into fully functional neutrophils [44]. Therefore, I examined the *gcsfr* mRNA levels in progenitor cells and purified kidney neutrophils. Consistent with this idea, the level of *gcsfr* mRNA increased from progenitor cells to kidney neutrophils. However, due to the low numbers of neutrophils in the blood and spleen, I was unable to generate a highly pure population of neutrophils from the peripheral blood or spleen, and thus, could not assess the levels of *gcsfr* mRNA in blood and spleen neutrophils. The presence of *gcsfr* mRNA in the progenitor cell population was of interest, as this further supported the idea that sorted R1 cells in PKM cultures contained a mixed population of hematopoietic stem/progenitor cells, macrophage-committed progenitors, and possibly granulocytic-committed progenitor cells [24]. When I examined *gcsfr* mRNA levels in cells from PKM cultures over time, I saw a decrease in the *gcsfr* mRNA over time. However, when I examined sorted progenitor cells from the PKM cultures over time, I did not observe a decrease in the level of *gcsfr* mRNA. This further supports my previous findings that, although a mixture of HPCs is isolated by our PKM culture methods, a specific population of monocyte/macrophage committed progenitor cells is being selectively acted upon by endogenous growth factors such as colony-stimulating factor-1 (CSF-1) to give rise to the monocytes and macrophages present in PKM cultures [45]. However, these results also provide novel insight into the presence and possible

maintenance of a population of granulocyte-committed progenitor cells, as demonstrated by the stable *gcsfr* mRNA levels in the sorted progenitor cells over time of culture.

Lastly, I wanted to examine how the mRNA levels of *gcsfr* could be modulated in goldfish neutrophils in response to a cocktail of mitogens or a natural pathogen of fish, heat-killed *A. salmonicida* A449. Both PKC activation and increased Ca^{2+} concentrations are known to activate mammalian neutrophils [46] and I have previously shown that PMA and calcium ionophore are capable of inducing respiratory burst and degranulation in goldfish neutrophils [12]. Similarly, these goldfish neutrophils degranulate and produce reactive oxygen intermediates in response to live *A. salmonicida* A449 [12]. Interestingly, I observed *gcsfr* mRNA levels to be up-regulated in neutrophils treated with either mitogens or heat-killed *A. salmonicida* A449. The up-regulation of *gcsfr* mRNA during neutrophil activation may be important to promote the survival of these cells through GCSF signaling as opposed to an apoptosis pathway [47].

However, mitogens and *A. salmonicida* appear to activate neutrophils in a different manner based on the unique modulation of the mRNA levels of goldfish *kita* and *prominin*. Goldfish *kita* [48] (Chapter 4) and *prominin* [49] have been previously cloned and characterized. Goldfish *kita* was selected based on the role of the KITA and KITLA in the survival of goldfish progenitor cells [48] and mammalian HPCs, as well as the ability of c-KIT^+ lin^- cells to give rise to neutrophils in the bone marrow and at the site of inflammation [50]. Although the levels of goldfish *kita* mRNA were initially low in kidney neutrophils, treatment of neutrophils with mitogens significantly down-regulated *kita* mRNA levels while treatment with heat-killed *A. salmonicida* A449 up-regulated *kita* mRNA levels.

Activation of progenitor cells is known to trigger differentiation [51], and therefore, activation of goldfish neutrophils with mitogens may explain the decrease in *kita* mRNA, as c-KIT protein is not expressed in mature mammalian neutrophils [50, 52]. However, in the case of *kita* mRNA levels increasing with *A. salmonicida* treatment, I can hypothesize that perhaps signaling through KITA may provide additional survival signals to the neutrophil, or it may be involved in the process of phagocytosis of the bacteria. The role of KITA in goldfish neutrophils in response to heat-killed *A. salmonicida* remains to be fully elucidated. Goldfish *prominin* mRNA was shown to have high expression in progenitor cells and macrophages, with lower expression in monocytes [49], and prominin has been associated with membrane domains such as such as the plasma membrane protrusions in stem cells [53], the microvilli of epithelial cells [53, 54], and the plasma membrane invaginations of rod photoreceptor cells [55], however, the function of prominin is unknown. It is interesting to note that the mRNA levels of *prominin* did not change in neutrophils in response to mitogens, but did significantly increase in neutrophils treated with heat-killed *A. salmonicida* A449. This up-regulation of *prominin* mRNA levels may also be related to the phagocytic response towards *A. salmonicida* A449 and the membrane pseudopodia that form during a phagocytic response.

The experiments presented is this chapter represent the first report on the distribution and modulation of *gcsfr* mRNA in teleosts. The predicted structure of goldfish GCSFR contains many of the key domains found within other GCSFRs important for ligand binding and intracellular signaling. The results show that *gcsfr* is highly expressed in the kidney and that mRNA message for *gcsfr* is enriched in a purified population of neutrophils from the kidney. The level of *gcsfr*

mRNA expression appears to increase in populations of cells where “mature” neutrophils are present. Expression of *gcsfr* mRNA was also observed in the population of kidney progenitor cells, and these progenitors may be maintained in culture in the absence of cell appropriate differentiation signals. I believe *gcsfr* mRNA may be used as a marker of neutrophils and their progenitors in the goldfish, and that the expression of *gcsfr* mRNA may be differentially modulated in response to mitogens or prokaryotic pathogens. Lastly, in addition to being the site of granulopoiesis, the goldfish kidney appears to house a relatively large population of functional neutrophils, similar to the reservoir of neutrophils in the mammalian bone marrow.

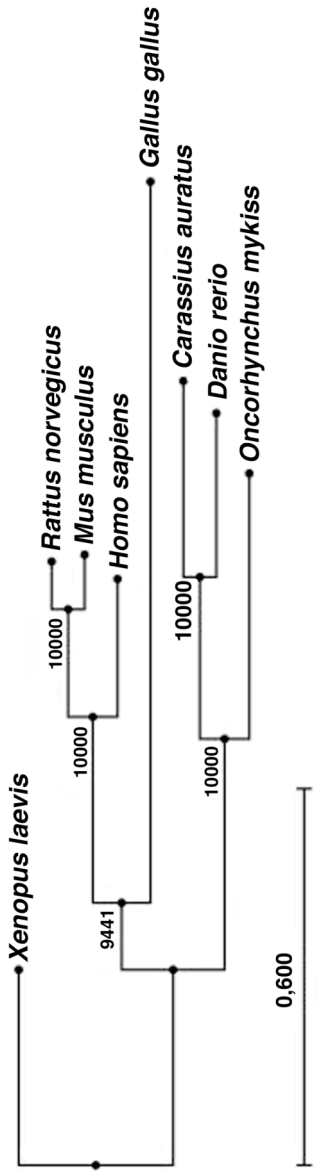


Figure 8.1: Phylogenetic analysis of goldfish *gcsfr*.
Goldfish *gcsfr* grouped closely with zebrafish *gcsfr*. The phylogenetic tree was bootstrapped 10000 times and expressed as a percentage. Accession numbers for sequences are as follows: *H. sapiens* NM_000760, *M. musculus* NM_007782, *R. norvegicus* NM_001106685, *G. gallus* NM_001030898, *X. laevis* XM_002938741, *O. mykiss* NM_001124402, *D. rerio* NM_001113377, and *C. auratus* (JF922012).

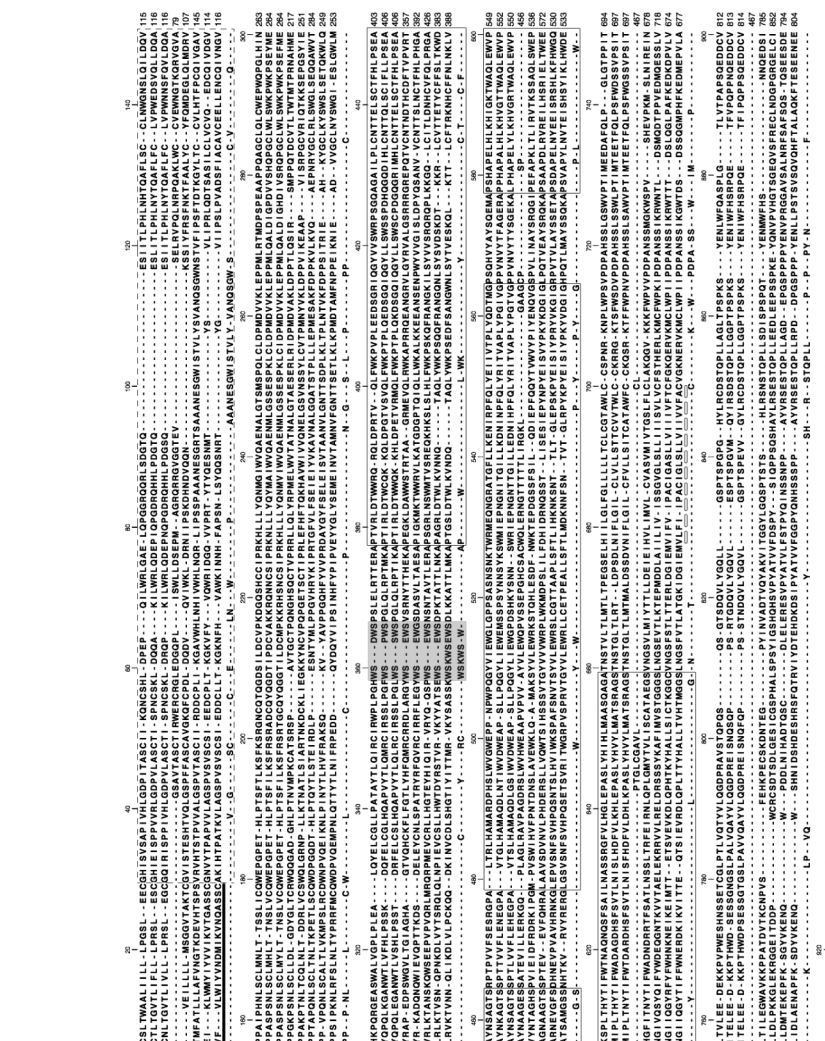


Figure 8.2: Amino acid alignment of GCSFR

Amino acids that are conserved in all sequences are shown below the aligned sequences. The signal peptide for goldfish GCSFR is underlined in black, the Ig-like domain is indicated by a dashed box, the consensus WSXWS motif is indicated with a shaded grey box, the three fibronectin (FN) domains are shown in open boxes, and the predicted transmembrane region is denoted with a open dashed bar. Accession numbers for sequences are as follows: *H. sapiens* NM_000760, *M. musculus* NM_007782, *R. norvegicus* NM_001106685, *G. gallus* NM_001030898, *X. laevis* XM_002938741, *O. mykiss* NM_001124402, *D. rerio* NM_001113377, and *C. auratus* (JF922012).

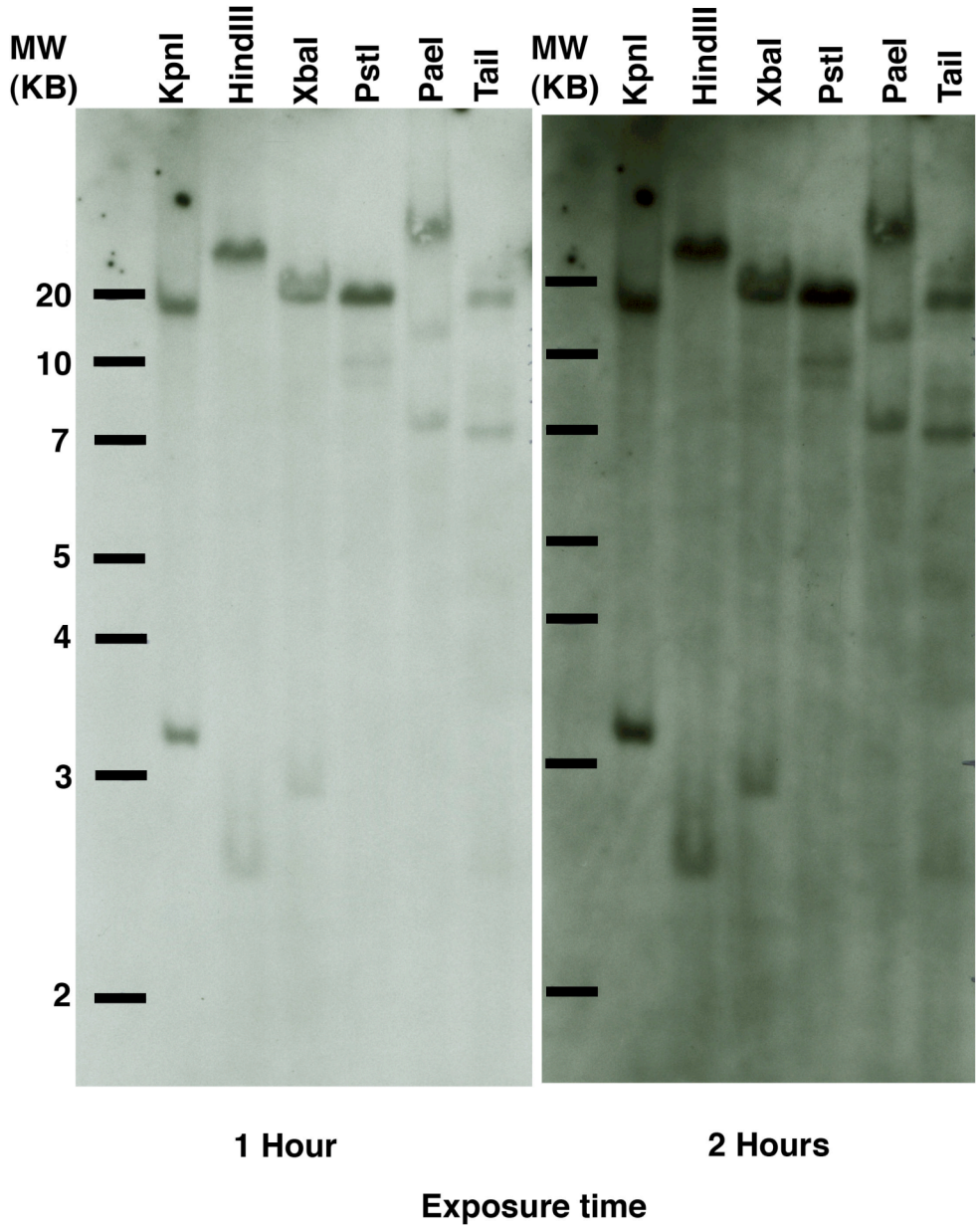


Figure 8.3: Southern blot for goldfish *gcsfr*.
Genomic DNA was isolated from goldfish blood and digested with restriction enzymes overnight. Digested DNA was run on a 0.8% agarose gel and transferred to a nylon membrane. The nylon membrane was probed overnight, washed and developed. The membrane was exposed to film for 1 hour (left panel) or 2 hours (right panel) to detect where the probe bound.

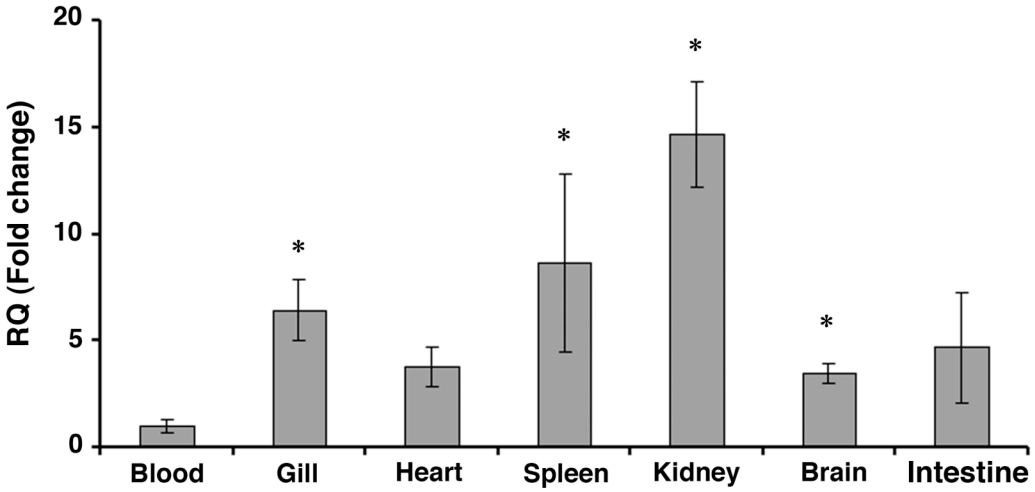


Figure 8.4: Expression of goldfish *gcsfr* in different goldfish tissues as determined by quantitative PCR.
Goldfish *gcsfr* mRNA levels were highest in kidney, spleen, gill and brain with lower expression in the heart, intestine and blood. Data was normalized to the blood, mean ± standard error is shown (n = 4). Significance is denoted by (*) compared to the blood reference sample ($P < 0.05$).

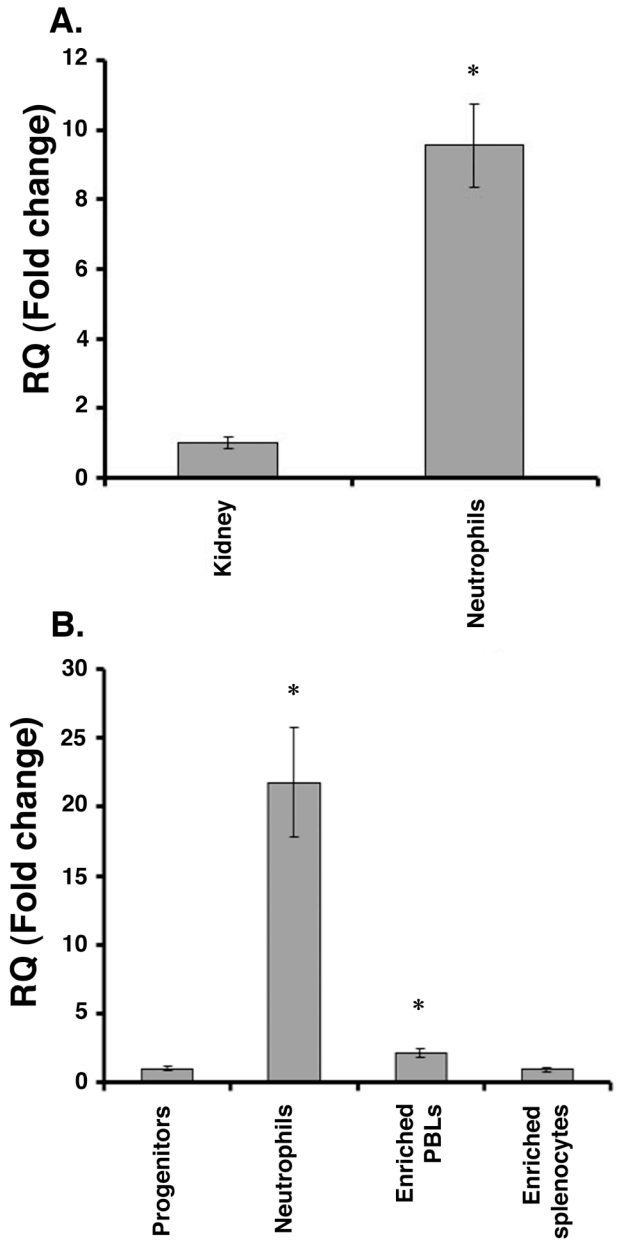


Figure 8.5: Comparison of the expression of goldfish *gcsfr* in cell populations using quantitative PCR.
(A) Neutrophils from 4 individual fish were seeded in complete NMGFL-15 medium overnight prior to RNA isolation. Data was normalized to kidney tissue from four individual fish and the mean ± standard error (n = 4) is shown. (B) Progenitor cells were sorted from day 0 primary kidney macrophage cultures, neutrophils isolated from kidney tissue, or enriched for neutrophils from peripheral blood leukocytes (PBLs) or splenocytes isolated on a step-wise Percoll gradient (51%-60%-75%). The mean ± standard error is shown (n = 3-4). Significance is denoted by (*) compared to the progenitor cell reference sample ($P < 0.05$).

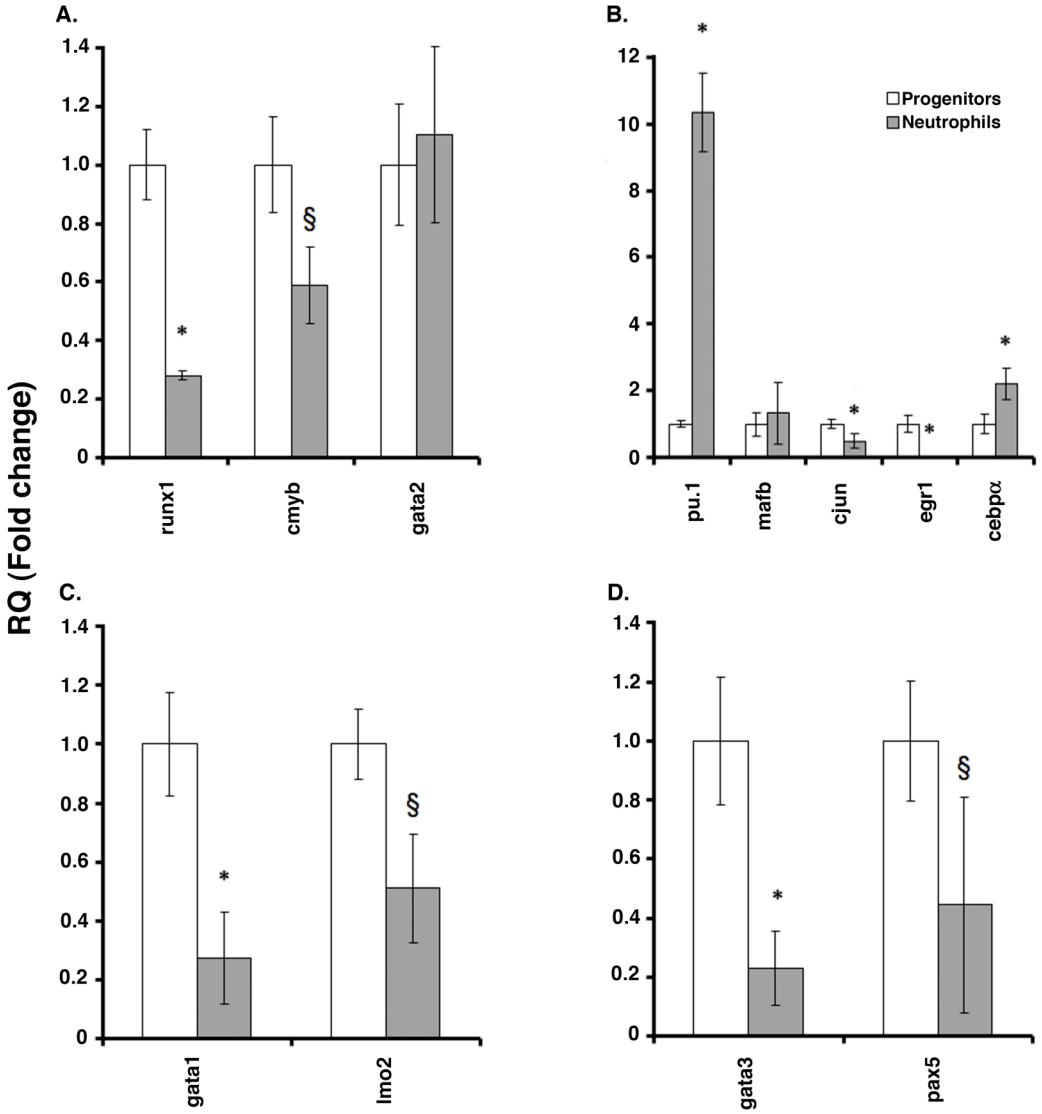


Figure 8.6: Comparisons of expressions of goldfish hematopoietic transcription factors in goldfish progenitor cells and neutrophils using quantitative PCR.

Quantitative PCR expressions of goldfish transcription factors involved in early hematopoiesis (A), myelopoiesis (B), erythropoiesis (C), and lymphopoiesis (D) in goldfish progenitor cells and neutrophils. Data were normalized to the progenitor cells and standard error is shown. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§), (n = 4).

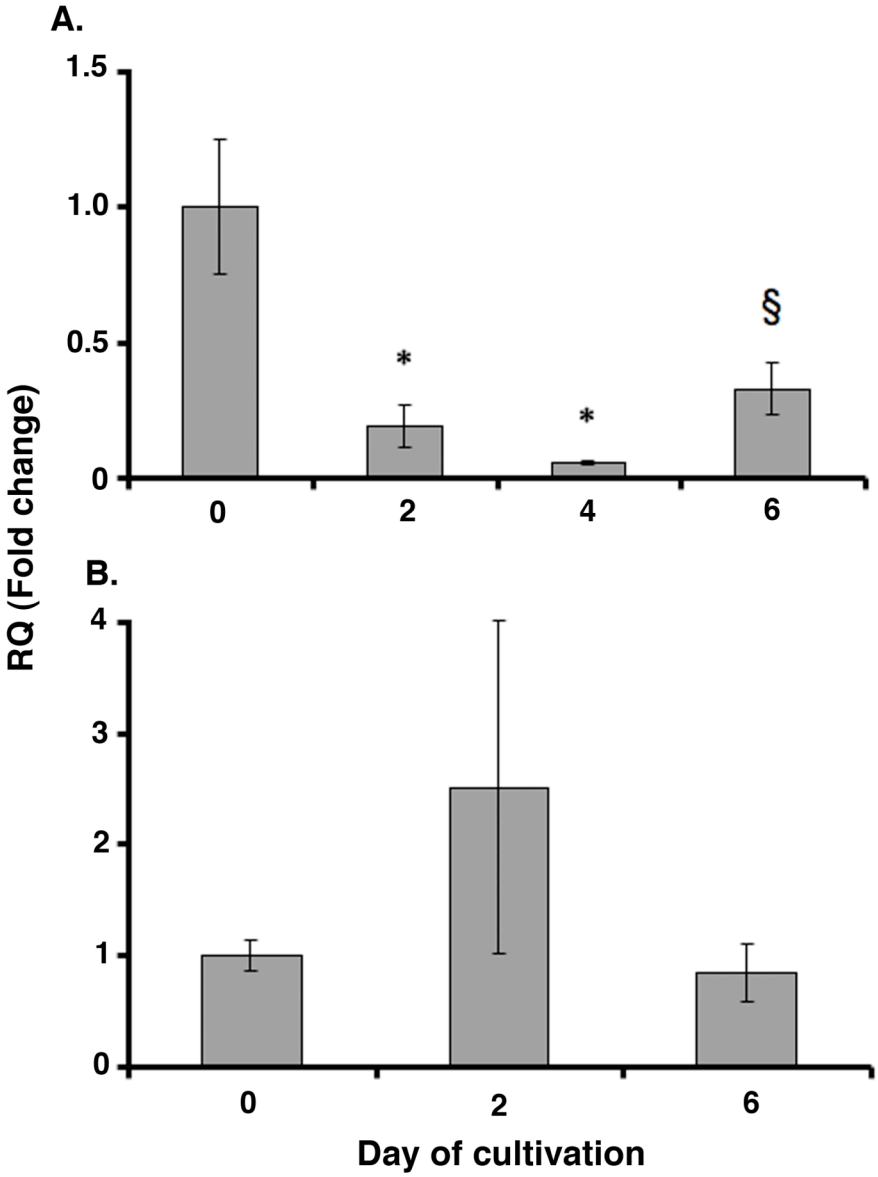


Figure 8.7: Expressions of goldfish *gcsfr* in primary kidney macrophages (PKM) and sorted progenitors from various days of culture as determined by quantitative PCR.

(A) 2 x 10^6 PKM cells were seeded into a 6 well plate, and cells harvested at 0 days, 2 days, 4 days, and 6 days after cultivation. Data was normalized to the day 0 PKM reference sample and error bars are shown (n = 3). (B) Progenitor cells were sorted from PKM cultures after 0 days, 2, days and 6 days of culture. Data was normalized to the day 0 sorted progenitor cells and standard error is shown (n = 4). Significance of $P < 0.05$ is denoted by (*) and $P < 0.08$ is denoted by (§), compared to the reference sample.

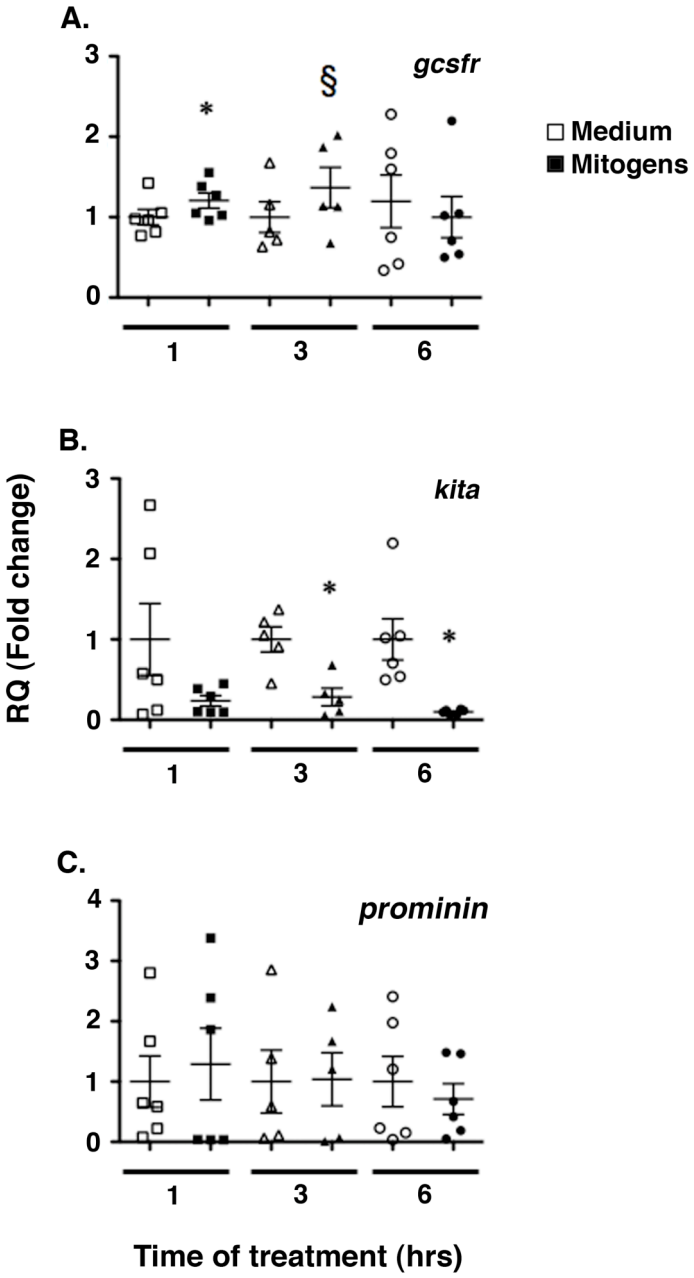


Figure 8.8: Expressions of goldfish *gcsfr*, *kita* and *prominin* in neutrophils treated with mitogens as determined using quantitative PCR.
Quantitative PCR of goldfish *gcsfr* (A), *kita* (B) and prominin (C) mRNA levels. Two million neutrophils were treated with medium (negative control) or a cocktail of mitogens (PMA, CaI, ConA) for 1, 3, or 6 hrs. Data was normalized to the time-matched medium-treated neutrophil controls. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§), (n = 5-6).

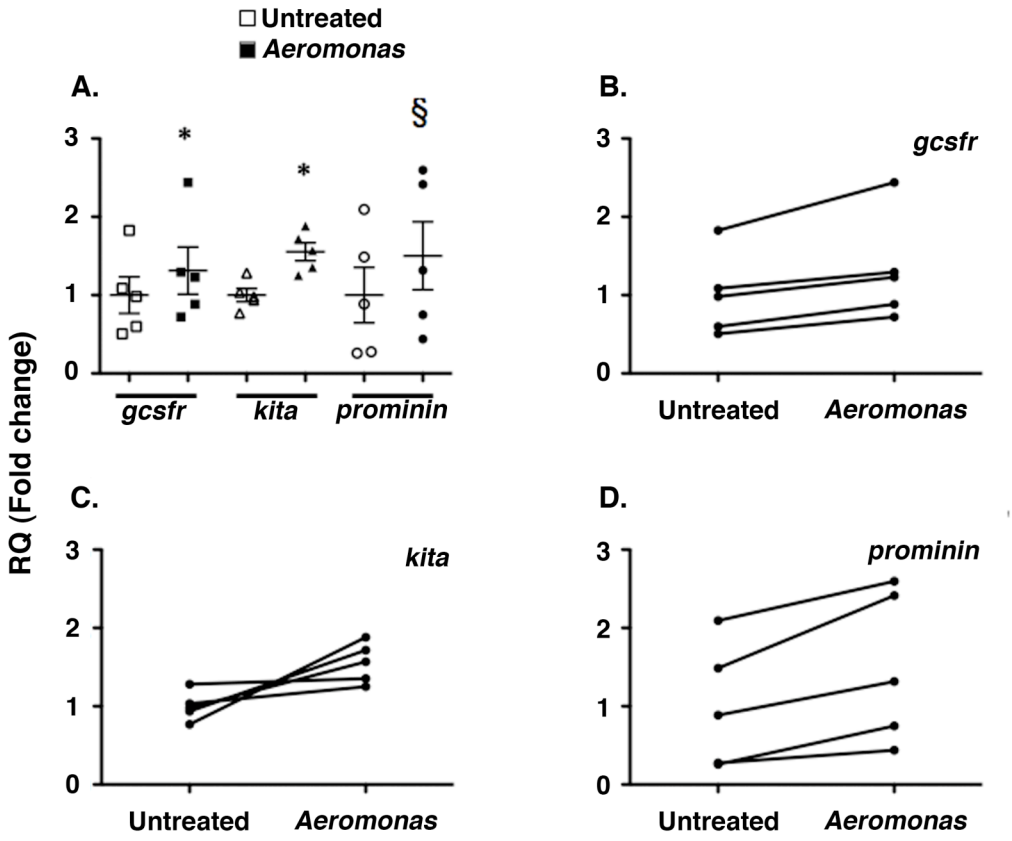


Figure 8.9: Quantitative PCR of goldfish *gcsfr, kita,* and *prominin* in neutrophils treated with heat-killed *A. salmonicida* A449.
Two million neutrophils were seeded in a 6-well plate and treated with medium (negative control) or heat-killed *A. salmonicida* A449 for 3 hrs prior to RNA isolation. (A) Data for *gcsfr*, *kita*, and *prominin* is shown as a mean + standard error. To demonstrate the increase in mRNA levels of *gcsfr* (B), *kita* (C), and *prominin* (D), individual data points and their corresponding levels after *A. salmonicida* A449 treatment are connected with a line. Significance is denoted by (*) compared to the reference sample, $P < 0.05$, and $P < 0.08$ is denoted by (§), (n = 5).

8.4 References

[1] **Hallett MB, Dewitt S.** Ironing out the wrinkles of neutrophil phagocytosis. Trends Cell Biol. 2007;17(5):209-14.

[2] **Nathan C.** Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006;6(3):173-82.

[3] **Pham CT.** Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol. 2006;6(7):541-50.

[4] **Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al.** Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

[5] **Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al.** Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem. 2004;279(42):44123-32.

[6] **Witko-Sarsat V, Pederzoli-Ribeil M, Hirsh E, Sozzani S, Cassatella MA.** Regulating neutrophil apoptosis: new players enter the game. Trends Immunol. 2011;32(3):117-24.

[7] **Panopoulos AD, Watowich SS.** Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. Cytokine. 2008;42(3):277-88.

[8] **Liongue C, Wright C, Russell AP, Ward AC.** Granulocyte colony-stimulating factor receptor: stimulating granulopoiesis and much more. Int J Biochem Cell Biol. 2009;41(12):2372-5.

[9] **Richards MK, Liu F, Iwasaki H, Akashi K, Link DC.** Pivotal role of granulocyte colony-stimulating factor in the development of progenitors in the common myeloid pathway. Blood. 2003;102(10):3562-8.

[10] **Avalos BR.** Molecular analysis of the granulocyte colony-stimulating factor receptor. Blood. 1996;88(3):761-77.

[11] **Shimoda K, Okamura S, Harada N, Niho Y.** Detection of the granulocyte colony-stimulating factor receptor using biotinylated granulocyte colony-stimulating factor: presence of granulocyte colony-stimulating factor receptor on CD34-positive hematopoietic progenitor cells. Res Exp Med (Berl). 1992;192(4):245-55.

[12] **Katzenback BA, Belosevic M.** Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney. Dev Comp Immunol. 2009;33(4):601-11.

[13] **Harun NO, Zou J, Zhang YA, Nie P, Secombes CJ.** The biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. Dev Comp Immunol. 2008;32(6):673-81.

[14] **Overland HS, Pettersen EF, Ronneseth A, Wergeland HI.** Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 2010;28(1):193-204.

[15] **Mayumi M, Takeda Y, Hoshiko M, Serada K, Murata M, Moritomo T, et al.** Characterization of teleost phagocyte NADPH oxidase: molecular cloning and expression analysis of carp (*Cyprinus carpio*) phagocyte NADPH oxidase. Mol Immunol. 2008;45(6):1720-31.

[16] **Palic D, Andreasen CB, Menzel BW, Roth JA.** A rapid, direct assay to measure degranulation of primary granules in neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). Fish Shellfish Immunol. 2005;19(3):217-27.

[17] **Palic D, Ostojic J, Andreasen CB, Roth JA.** Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol. 2007;31(8):805-16.

[18] **Palic D, Andreasen CB, Ostojic J, Tell RM, Roth JA.** Zebrafish (*Danio rerio*) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. J Immunol Methods. 2007;319(1-2):87-97.

[19] **Liongue C, Hall CJ, O'Connell BA, Crosier P, Ward AC.** Zebrafish granulocyte colony-stimulating factor receptor signaling promotes myelopoiesis and myeloid cell migration. Blood. 2009;113(11):2535-46.

[20] **Ahmed F, Kumar M, Raghava GP.** Prediction of polyadenylation signals in human DNA sequences using nucleotide frequencies. In Silico Biol. 2009;9(3):135-48.

[21] **Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, et al.** Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. Blood. 1990;75(4):851-7.

[22] **Budel LM, Touw IP, Delwel R, Lowenberg B.** Granulocyte colony-stimulating factor receptors in human acute myelocytic leukemia. Blood. 1989;74(8):2668-73.

[23] **Hanazono Y, Hosoi T, Kuwaki T, Matsuki S, Miyazono K, Miyagawa K, et al.** Structural analysis of the receptors for granulocyte colony-stimulating factor on neutrophils. Exp Hematol. 1990;18(10):1097-103.

[24] **Katzenback BA, Karpman M, Belosevic M.** Distribution and expression analysis of transcription factors in tissues and progenitor cell populations of the goldfish (*Carassius auratus* L.) in response to growth factors and pathogens. Mol Immunol. 2011.

[25] **Neumann NF, Barreda D, Belosevic M.** Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[26] **Neumann NF, Barreda DR, Belosevic M.** Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[27] **Anaguchi H, Hiraoka O, Yamasaki K, Naito S, Ota Y.** Ligand binding characteristics of the carboxyl-terminal domain of the cytokine receptor homologous region of the granulocyte colony-stimulating factor receptor. J Biol Chem. 1995;270(46):27845-51.

[28] **Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y, Nagata S.** Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 1991;10(10):2855-65.

[29] **Santos MD, Yasuike M, Hirono I, Aoki T.** The granulocyte colony-stimulating factors (CSF3s) of fish and chicken. Immunogenetics. 2006;58(5-6):422-32.

[30] **Risinger C, Larhammar D.** Multiple loci for synapse protein SNAP-25 in the tetraploid goldfish. Proc Natl Acad Sci U S A. 1993;90(22):10598-602.

[31] **Luo J, Lang M, Salzburger W, Siegel N, Stolting KN, Meyer A.** A BAC library for the goldfish *Carassius auratus auratus* (Cyprinidae, Cypriniformes). J Exp Zool B Mol Dev Evol. 2006;306(6):567-74.

[32] **Tse DL, Chow BK, Chan CB, Lee LT, Cheng CH.** Molecular cloning and expression studies of a prolactin receptor in goldfish (*Carassius auratus*). Life Sci. 2000;66(7):593-605.

[33] **Diederich K, Schabitz WR, Minnerup J.** Seeing old friends from a different angle: Novel properties of hematopoietic growth factors in the healthy and diseased brain. Hippocampus. 2010.

[34] **Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE.** Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood. 2001;98(10):3087-96.

[35] **Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG.** Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A. 1997;94(2):569-74.

[36] **Hosoya T, Maillard I, Engel JD.** From the cradle to the grave: activities of GATA-3 throughout T-cell development and differentiation. Immunol Rev. 2010;238(1):110-25.

[37] **Loose M, Swiers G, Patient R.** Transcriptional networks regulating hematopoietic cell fate decisions. Curr Opin Hematol. 2007;14(4):307-14.

[38] **Fuxa M, Busslinger M.** Reporter gene insertions reveal a strictly B lymphoid-specific expression pattern of Pax5 in support of its B cell identity function. J Immunol. 2007;178(12):8222-8.

[39] **Brandt SJ, Koury MJ.** Regulation of LMO2 mRNA and protein expression in erythroid differentiation. Haematologica. 2009;94(4):447-8.

[40] **Burda P, Laslo P, Stopka T.** The role of PU.1 and GATA-1 transcription factors during normal and leukemogenic hematopoiesis. Leukemia. 2010;24(7):1249-57.

[41] **Cai DH, Wang D, Keefer J, Yeamans C, Hensley K, Friedman AD.** C/EBP alpha:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBP alpha homodimers or AP-1. Oncogene. 2008;27(19):2772-9.

[42] **Krishnaraju K, Hoffman B, Liebermann DA.** Early growth response gene 1 stimulates development of hematopoietic progenitor cells along the macrophage lineage at the expense of the granulocyte and erythroid lineages. Blood. 2001;97(5):1298-305.

[43] **Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG.** PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. Blood. 1996;88(4):1234-47.

[44] **Tsuji K, Ebihara Y.** Expression of G-CSF receptor on myeloid progenitors. Leuk Lymphoma. 2001;42(6):1351-7.

[45] **Hanington PC, Hitchen SJ, Beamish LA, Belosevic M.** Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. Fish Shellfish Immunol. 2009;26(1):1-9.

[46] **Sayeed MM.** Exuberant Ca(2+) Signaling in Neutrophils: A Cause for Concern. News Physiol Sci. 2000;15130-6.

[47] **Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR.** Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature. 1990;343(6253):76-9.

[48] **Katzenback BA, Belosevic M.** Molecular and functional characterization of kita and kitla of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2009;33(11):1165-75.

[49] **Walsh JG, Barreda DR, Belosevic M.** Cloning and expression analysis of goldfish (*Carassius auratus* L.) prominin. Fish Shellfish Immunol. 2007;22(4):308-17.

[50] **Kim MH, Granick JL, Kwok C, Walker NJ, Borjesson DL, Curry FR, et al.** Neutrophil survival and c-kit^{+}-progenitor proliferation in *Staphylococcus aureus*-infected skin wounds promote resolution. Blood. 2011;117(12):3343-52.

[51] **Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, et al.** Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity. 2006;24(6):801-12.

[52] **Broudy VC, Lin N, Zsebo KM, Birkett NC, Smith KA, Bernstein ID, et al.** Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. Blood. 1992;79(2):338-46.

[53] **Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, et al.** The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem. 2000;275(8):5512-20.

[54] **Corbeil D, Roper K, Hannah MJ, Hellwig A, Huttner WB.** Selective localization of the polytopic membrane protein prominin in microvilli of epithelial

cells - a combination of apical sorting and retention in plasma membrane protrusions. J Cell Sci. 1999;112 (Pt 7)1023-33.

[55] **Maw MA, Corbeil D, Koch J, Hellwig A, Wilson-Wheeler JC, Bridges RJ, et al.** A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. Hum Mol Genet. 2000;9(1):27-34.

Chapter 9: General discussion

9.1 Overview of findings

Macrophages and neutrophils are the sentinel cells of the innate immune response of vertebrates, such as teleosts. As phagocytic myeloid cells, they are involved in homeostatic mechanisms, wound healing, and the detection, elimination and clearance of foreign entities including tumors, virus-infected cells and invading pathogens. Furthermore, macrophages and neutrophils are also responsible for the production of hundreds of bioactive molecules. These bioactive molecules are important in pathogen recognition and destruction, cellular communication and activation, initiation of an adaptive immune response and later, resolution of an inflammatory response and tissue repair. Clearly, neutrophils and macrophages are essential to the survival of vertebrates.

However, all good things must come to an end; macrophages and neutrophils, the latter in particular, have a finite lifespan. Therefore, a manufacturing centre, the hematopoietic niche, is essential for the production of myeloid cells. The hematopoietic niche must maintain basal myeloid cell production levels during homeostasis, yet retain the flexibility to ramp-up myeloid cell production in response to physiological demands, such as during a pathogenic insult. Together, the development of macrophages (monopoiesis) and neutrophils (granulopoiesis) is collectively known as myelopoiesis, and is regulated by the complex interaction of CSFs, their receptors, and intracellular

transcription factor machinery that control lineage fate decisions and terminal differentiation events.

Within the field of comparative immunology, a large body of work has surrounded the characterization of teleost cytokines and receptors involved in inflammation and their cellular targets (primarily macrophages). In comparison, relatively little is known about the mechanisms that govern the production of these myeloid cells. Research on teleost myelopoiesis is hampered by the lack of reagents, the difficulty in isolating appreciable numbers of relatively pure populations of HSCs/HPCs, and in identifying key growth factors important for myeloid cell development due to evolutionary selection pressures. Therefore, the central focus of my thesis was to further our understanding of the surface receptors, growth factors and transcription factors important in maintaining progenitor cells and in driving their differentiation along a macrophage or neutrophil lineage, using the goldfish (*Carassius auratus* L.) as a model system.

The major hematopoietic organ of teleosts is the kidney, akin to that of mammalian bone marrow. Recent studies in the cyprinids *Danio rerio* and *Carassius auratus langsdorfii* demonstrated that residing within the kidney are HSCs and HPCs capable of giving rise to all blood cell lineages, including the myeloid lineage [1-8]. Previous studies in the Belosevic laboratory have established a goldfish PKM culture system for the *in vitro* study of teleost monopoiesis. Through a series of experiments, it was shown that small mononuclear cells isolated from the kidney spontaneous proliferate and differentiate into monocytes and macrophages, driven by the production of their own endogenous growth factors [9, 10], such as CSF-1 [11, 12]. This is in contrast to the mammalian systems that require the addition of exogenous

growth factors to drive macrophage differentiation. Furthermore, goldfish PKM cultures retained all three populations (progenitors, monocytes and macrophages) in later stages of *in vitro* culture [9, 10]. Upon comparison of the cyprinid model systems, and work performed during this thesis, it became apparent that the freshly isolated progenitor cells likely comprised of a mixture of HSCs, HPCs, and committed progenitor cells. However, when placed in culture, these progenitors only differentiated along the monocyte/macrophage lineage. This suggests the initial presence of a large population of committed macrophage progenitors, the commitment of HSPCs to the macrophage lineage in culture, or simply that the culture conditions were not permissive to the outgrowth of other cell lineages. Furthermore, the progenitor cell population persisted for the duration of cultivation, suggesting they must receive survival signals.

To begin to understand the regulatory mechanisms governing the survival, proliferation, differentiation and lineage fate decisions of myeloid progenitor cells, a multi-faceted approach is necessary. Therefore, I focused on examining the receptors, growth factors and transcription factors and their interactions that form the basis of the innate complexity governing myelopoiesis.

I first focused on identifying and characterizing *kita* and *kitla* (Chapter 4) since their mammalian counterparts are implicated in the biology of early progenitor cells. *In silico* analysis of goldfish KITA showed structural similarity to mammalian KIT proteins, with the exception of the loss of an Ig domain in the extracellular domain. Based on the *kita* expression studies, I concluded that *kita* was primarily a marker of fish progenitor cells. However, *kita* mRNA was still detected in monocytes and macrophages and was upregulated in day 8 PKMs when treated with *A. salmonicida*. This suggested that teleost *kita* was retained at

low levels in monocytes and macrophages and may play a role during the activation of these phagocytes. The rgKITLA was glycosylated, forming homodimers and tetramers in cross-linking experiments. While the formation of homodimers has been reported for mammalian SCFs, the formation of KITLA tetramers has not been reported in any system, and may be unique to teleosts. The formation of KITLA tetramers in teleosts may be a mechanism to regulate the intensity of receptor signaling. However, the function of KITLA dimers versus tetramers requires further study. Through a series of functional experiments, I demonstrated the role of rgKITLA in promoting chemotaxis, proliferation and maintenance of goldfish progenitor cells. Interestingly, rgKITLA was not only chemoattractive to day 2 PKMs consisting of mainly progenitors, but to day 6-9 PKMs consisting of mainly mature macrophages as well. This coincides with the low level of *kita* mRNA observed in macrophages that may be translated into functional KITA on the surface of monocytes/macrophages. Alternatively, it may suggest that goldfish KITLA is able to signal through another receptor to promote the chemotaxis of macrophages.

As GMPs restrict to committed macrophage progenitors, subsequently differentiating into monocytes and macrophages, they express CSF-1R [13]. Consequently, the surface expression of CSF-1R has been used to identify members of the mononuclear phagocyte system in higher vertebrates [14, 15]. Although CSF-1R has been reported on teleost monocytes and macrophages [16-18], the presence of CSF-1R on goldfish progenitor cells had not been reported. Therefore, my efforts were directed towards determining if the PKM progenitors that give rise to monocytes and macrophages expressed CSF-1R on their surface (Chapter 5). Using an antibody to sCSF-1R, a population of CSF-

1R$^+$cells was identified in different goldfish tissues. Examination of PKMs demonstrated a population of CSF-1R$^+$ cells within the progenitor, monocyte, and macrophage flow cytometer gates. Interestingly, a large population of freshly isolated progenitors were CSF-1R$^+$, and this CSF-1R$^+$ population declined with time of cultivation, concomitant with the generation of monocytes and macrophages. These data represented the first report of CSF-1R$^+$ progenitor cells and suggested that CSF-1R protein expression may be a maker for cells of the mononuclear phagocyte system in teleosts.

The expression and relative level of distinct transcription factors are important at multiple junctures of developing immune cells and during the development of multiple cell lineages. As such, the expression of TFs can be used as markers of cell lineage [19], and have been utilized to identify different lineages in the zebrafish model system. I surveyed the expression of a panel of TFs in tissues, various cell populations, and in progenitor cells in response to treatment with myeloid growth factors or in progenitor cells isolated from *in vivo* challenged goldfish (Chapter 6). Based on the expression of TFs in a variety of tissues and cell populations, I concluded the following: (1) the expression of TFs appeared to be representative of the cell types present in tissues and cell populations, (2) the group of TFs important for progenitor cell differentiation into the monocyte/macrophage lineage is largely consistent with the regulation of transcription factors in mammalian macrophage development, (3) during cultivation of PKM progenitor cells, the changes in TF expression are likely representative of lineage commitment events, (4) treatment of progenitor cells with growth factors modulate TF expression consistent with the previously characterized functions of the growth factors, and (5) TF mRNA levels in

progenitors can be differentially modulated in vivo upon challenge with pathogens. During the progression of these studies, it became apparent that the expression of TFs was largely conserved between higher and lower vertebrates. However, there were also differences between teleosts and mammals. These included differences in TF expression amongst progenitors, monocytes and macrophages, and in the regulation of TF expression in progenitor cells when treated with rgCSF-1, when compared to that of the mammalian system. These differences suggest that while the final outcome of monopoiesis (production of macrophages) is conserved, the mechanisms that govern this process may be divergent.

Studies in the goldfish system up until this point have focused on characterizing the development of macrophages and the soluble factors that mediate their proliferation, differentiation, and activation. However, in order to begin to identify the soluble factors, receptors and transcription factors involved granulopoiesis, the isolation and characterization of goldfish neutrophils was required (Chapter 7). An isolation protocol was developed to obtain a highly pure (>92%) population of primary kidney neutrophils. Neutrophils were identified and characterized based on the following: (1) positive staining for myeloperoxidase, Sudan black and acid phosphatase, (2) expression of *mpo* but not *csf-1r*, and lack of binding by the anti-sCSF-1R (3) production of reactive oxygen intermediates to mitogens and *A. salmonicida* (4) capacity to degranulate in response to mitogens and *A. salmonicida*, and (5) a chemotactic response to *A. salmonicida* and *A. salmonicida* conditioned supernatants. Similar to other reports on the longer life span of neutrophils in other teleosts compared to mammals, I found that goldfish neutrophils were viable for at least four days in

culture and were capable of producing reactive oxygen intermediates. These data suggested that a large reservoir of functionally competent neutrophils exists in the goldfish kidney, similar to that of the bone marrow of mammals. Although goldfish neutrophils do not appear to possess a segmented nucleus that is characteristic of neutrophils of higher vertebrates, they appear to be the functional equivalent to their mammalian counterparts.

Following the characterization of goldfish neutrophils, the characterization of the receptor (GCSFR) and transcription factors involved in teleost neutrophil development (Chapter 8) was required. *In silico* analysis showed goldfish GCSFR retained the conserved extracellular and intracellular domains of a type I cytokine receptor. The *gcsfr* showed the highest expression in the hematopoietic tissues, and in the purified neutrophil population. The expression of *gcsfr* in neutrophils was upregulated in response to mitogens and heat-killed *A. salmonicida*. Interestingly, I also observed the differential regulation of *kita* and *prominin* expression when neutrophils were treated with mitogens or pathogens in a time dependent manner. While the treatment of neutrophils with mitogens functioned to down-regulate the expression of *kita*, treatment with heat-killed *A. salmonicida* up-regulated the expression of *kita*. The up-regulation of *kita* in response to *A. salmonicida* was also observed in macrophages (Chapter 4). These data point to a conserved role for *kita* expression in phagocytes in response to bacteria, and suggest that *kita* may play a novel role in phagocytosis, although future work is required to elucidate this role. Lastly, I found that neutrophils displayed increased mRNA levels of TFs associated with development along a granulocyte lineage, while TF mRNA levels associated with macrophage development were decreased, compared to sorted progenitors. This

was in contrast to the increase or retention of macrophage lineage associated TF mRNA levels in monocytes (Chapter 6). These data suggest that neutrophil and monocyte development are controlled by different groups of TFs, coinciding with studies performed in mouse and zebrafish.

9.2 Evolution of myelopoiesis in metazoans

With the evolution of multicellular metazoans from unicellular eukaryotes came the requirement for mechanisms to facilitate cell communication, interaction, and specification into diversified cell types required to perform specialized functions or comprise entire systems. A specialized system that evolved to recognize self from non-self as a defense mechanism against pathogenic insult was the innate immune system [20]. The central cell of the innate immune system is the phagocyte: capable of recognizing, engulfing (phagocytosis), and neutralizing pathogens. The process of phagocytosis for host defense in metazoans is thought to have evolved from phagocytosis for nutrient uptake in unicellular organisms [21].

All metazoans have some form of an innate immune response for defense against pathogens and all involve a phagocytic cell. Sponges, thought to represent the basal clade of the metazoans, posses phagocyte-like archaeocytes, [22]. Sea urchins have 3 types of phagocytes, based on morphology, as well as red spherules cells that rapidly appear at wound and infection sites, releasing the antimicrobial compounds contained within their granules [23]. The freshwater crayfish, *Pacifastacus leniusculus*, has been used as a model to study crustaceans and possesses three haemocyte

subpopulations. The first is the hyaline cells that are phagocytic, the semi-granular cells that contains a number of eosinophilic granules and retains the capacity to phagocytose, and lastly, the granular cells that are the key players in the melanization pathway, reviewed by [24]. *Drosophila* have three types of haemocytes: plasmatocytes, crystal cells and lamellocytes. The plasmatocytes are phagocytes capable of engulfing apoptotic bodies and pathogens. The crystal cells degranulate and are involved in melanization, an important insect response involved in immunity and in wound repair. Lastly, lamellocytes are only produced in a stress response and act to encapsulate pathogens that are too large to be phagocytosed [25]. As mentioned previously, macrophage and neutrophil are the primary phagocytic cells of the innate immune system of vertebrates. In all but the sponge, there appears to be a functional equivalent of the vertebrate monocyte/macrophage and granulocyte cell types. The presence of phagocytes in all metazoans suggests that the evolution of phagocytosis as a means of host defense may have been a single evolutionary event prior to the emergence of metazoans [22].

In order for metazoans to produce specialized innate immune cells, they must also have had to develop a means for the specification and differentiation of these cell types. Therefore, it stands to reason that prior to the production of distinct cell types by metazoans, they had to develop a number of cellular communication (cytokines and receptors) and signaling mechanisms (signal transducers, transcription factors). These molecules would be essential for the orchestration of transcription of distinct groups of genes, from identical DNA molecules contained within each cell, to produce the desired outcome, i.e. specialized cell types that were functionally diverse from one another. While

these basic principles, including the presence of a hematopoietic site containing prohaemocytes, are conserved in metazoans, the actual soluble mediators and much of the intracellular machinery appear to be divergent.

Studies on invertebrate myelopoiesis are limited. Perhaps the most studied invertebrate is that of *Drosophila*. Using the *Drosophila* model system multiple waves of myelopoiesis have been documented, arising first in the head mesoderm during the embryonic stage and later in the lymph gland in larval stages [25, 26]. However, no site of myelopoiesis has been identified in adult *Drosophila.* Instead, plasmatocytes from embryonic stage persist into the larval stage, and cell division occurs in the blood to give rise to a ten-fold increase in plasmatocytes, in addition to the production of larval plasmatocytes reviewed by [25, 26]. The maturation, migration and survival of plasmatocytes has been shown to be dependent on the receptor tyrosine kinase platelet derived growth factor / vascular endothelial growth factor receptor (PDGF/VEGF receptor; PVR). The three ligands of PVR, PVF 1-3, have been shown to promote the survival of plasmatocytes through anti-apoptotic mechanisms [26]. In terms of transcription factors, *Drosophila* have ancestral GATA, RUNX, and zinc finger transcription family members that play a role in cell fate decisions [26].

Studies in the crayfish *P. leniusculus* have shown that haemocytes need to be continually replaced throughout the life of the organism, and this progression of cell development from the prohaemocyte to differentiated cell types occurs in the hematopoietic tissue of adults. The soluble mediators of cell development are the novel group of astakines, AST1 and AST2. The astakines are the only known hematopoietic growth factors identified in crustaceans. Like that of Drosophila, GATA and RUNX transcription factors are found in *P.*

leniusculus, and appear to be important for haemocyte survival and specification, reviewed in [24]. However, like that of higher vertebrates, invertebrates appear to be able to increase myelopoiesis in response to wounding or parasitism, and this process can occur at the expense of other haemocyte lineages [24, 26].

Finally, sequencing of the purple sea urchin genome has revealed a number of transcription factor homologues to those known to be important in hematopoiesis of higher vertebrates including ETS (*pu.1, ets-1,2*), an ancestral GATA, RUNX, C/EBP, Zinc Finger (*egr-1,2,3*, *gfi-1*), interferon response factors (*irf-1,2,4,8*), bZIP (*c-jun*, *c-fos*, etc.), and MYB (*cmyb*) transcription family members, among others [27]. While two homologues of the VEGFR were detected from genome sequencing of the purple sea urchin, homologues for PDGFR, c-KIT, FLT3, or CSF-1R, or any of the hematopoietic growth factors were not identified [27].

The divergence of teleosts and mammals occurred approximately 400-450 Mya, thus teleosts represent one of the most basal groups of vertebrates [28]. Comparison of soluble factors and their receptors in teleosts and mammals show retention of many of important hematopoietic growth factors and receptors, including PDGFR [29-31], c-KIT [32-34], FLT3 (accession number DQ317446), CSF-1R [16, 18, 29, 30, 35, 36], GCSFR [37, 38], and their ligands PDGF [39], KIT ligand [32, 34, 40] , CSF-1 [12, 41], and GCSF [37, 42, 43], although FLK2, the ligand to FLT3, has not yet been reported. However, it appears that teleosts do not possess the hematopoietic growth factors IL-3 and GM-CSF, and their cognate receptors. In addition, teleosts possess all of the TF families required for hematopoiesis in higher vertebrates, reviewed in [44, 45]. Based on studies performed to date, the regulation of hematopoiesis is largely similar between

mammals and teleosts. However, teleosts often possess a number of gene duplications for many of the soluble factors, receptors, and to some extent, transcription factors as a result of a teleost-specific whole genome duplication predicted to have occurred approximately 350 Mya, and is believed to be responsible for the radiation of teleosts [46, 47]. Many of these teleost genes are rapidly evolving, often undergoing subfunctionalization or neofunctionalization, compared to mammalian genes, making the identification of teleost orthologues difficult.

To my knowledge, the soluble factors or transcriptional regulators governing the production of the phagocytes in sponges have not yet been reported. Therefore, it is difficult to speculate exactly when the regulatory elements of myelopoiesis arose. Based on the appearance of GATA, RUNX and zinc finger transcription factors and the VEGFR in *Drosophila* and a similar set of transcription factors in the crayfish, these transcription factors and receptor tyrosine kinase may represent primordial regulatory elements of myeloid cell development that arose in the common ancestor of protostomes and deuterostomes. After the divergence of proteostomes and deuterostomes, some 550 Mya, it appears there may have been an explosion of transcription factors in the deuterostomes, as shown by the many TF homologues in the purple sea urchin. However, at this time, it appears that the soluble growth factors and their cognate receptors were still limited to the receptor tyrosine kinase VEGFR, and it was not until the appearance of vertebrates that another explosion of diversity occurred to give rise to the many hematopoietic growth factors and receptors that govern hematopoiesis characteristic of vertebrates. However, as more genomic and functional data are gathered, it may reveal the presence of previously

unidentifiable homologues of hematopoietic factors due to the evolutionary selective pressures acting on them. One thing is clear, that with increased complexity of cellular communication and intracellular regulatory machinery, came the emergence of highly complex and efficient immune systems.

Although we can use comparative model systems to gain an understanding of the evolution of myelopoiesis, we also have to be cautious regarding our speculations, keeping in mind the large evolutionary distances that separate these model systems, each having undergone their own independent evolution since their divergence. Each organism is subject to distinct environments, pathogens, developmental stages and life spans, among a myriad of other factors. Therefore, their hematopoietic needs may differ in terms of cell types, their functions, and the particular soluble factors and intracellular transcription factors that regulate their development.

9.3 The goldfish (*Carassius auratus* L.) as a model system of teleost myelopoiesis.

Besides the goldfish, the major models of teleost hematopoiesis and myelopoiesis are the ginbuna carp and the zebrafish. The ginbuna carp was advantageous for *in vivo* transplantation and reconstitution experiments, demonstrating the existence of an HSC that generates all hematopoietic cells. Likewise, the zebrafish has also been used for transplantation studies, but by far the major advantage provided by zebrafish are the utility of gene knockdowns (morpholinos) and recently knockouts (zinc finger nucleases) due to the availability of genomic information. However, the zebrafish does not lend itself to

being developed as an *in vitro* model due to its small size and the difficulty of isolating cells. Therefore the goldfish PKM culture system represents a unique opportunity to study myelopoiesis *in vitro*.

Drawing from previous observations in the Belosevic group as well as the work performed during my thesis, I propose an integrated model of myelopoiesis in the goldfish. The isolation of kidney leukocytes is a mixture of a population of lymphocytes, HSCs, HPCs and committed myeloid progenitors (Chapter 6). The population of HSCs, HPCs, and committed myeloid progenitor cells likely express KITA on their surface (Fig. 9.1), through which KITLA mediates their survival and proliferation (Chapter 4). During initial cultivation, a population of cells, likely myeloid progenitors themselves, the proposed alternative macrophages or perhaps a population of activated T-cells, produces endogenous growth factors, such as CSF-1 that then acts on progenitors expressing CSF-1R (Fig. 9.1, Chapter 5). Based on immunofluorescent staining, the CSF-1R^{+} progenitors appear to make up a large population of isolated kidney leukocytes (Chapter 5). However, the production of endogenous growth factors is limited to macrophage specific hematopoietic factors, resulting in the cell death of lymphocytes and their precursors and coincides with the lag phase of culture. Through CSF-1R signaling, the committed myeloid progenitor cells undergo proliferation and terminal differentiation into monocytes and macrophages (Fig. 9.1), consistent with the proliferative phase of culture. However, the population of kidney leukocytes also contains a population of progenitors expressing *gcsfr,* which is important for the production of neutrophils (Fig. 9.1, Chapters 7 and 8). This population of granulocyte progenitors may be retained throughout cultivation due to KITA signaling, but does not differentiate into granulocytes due lack of GCSF

production that is required for terminal differentiation (Fig. 9.1). At the later stages of cultivation, the progenitor cell population and the mature macrophage population also negatively regulate macrophage development through the production of a soluble CSF-1R (sCSF-1R), consistent with the senescence phase.

These events of cellular survival, proliferation, lineage specification and differentiation are controlled on a transcriptional level (Chapter 6). Early progenitors likely express *c-myb*, *egr1* and *gata2* are important for maintenance of survival and blocking differentiation. KITLA may prevent progenitor cell differentiation by upregulating *egr1* and *mafb* expression (Chapter 6). Following day 2 of cultivation (lag phase), the accumulation of CSF-1 in the culture medium likely reaches a threshold level that results in the commitment and differentiation of progenitor cells along the macrophage lineage (Fig. 9.2). As progenitors differentiate into monocytes they increase *runx1* and *pu.1* expression, while maintaining expression of *mafb*, *cjun*, and *egr1*. Upon differentiating from monocytes to macrophages, they decrease the expression of *runx1*, *gata2*, *mafb*, *c-jun*, *egr1*, and *pu.1* (Fig. 9.2). Although we do not possess an *in vitro* cultivation system to generate goldfish neutrophils from committed progenitors, the isolation of goldfish neutrophils (Chapter 7) and comparison to sorted kidney progenitors suggests that the up-regulation of *pu.1* and *cebpa* expression and the down-regulation of *runx1*, *c-jun*, and *egr1* are important for neutrophil differentiation from progenitor cells (Fig 9.2). These goldfish kidney leukocytes likely contain a committed granulocyte progenitor, however, its differentiation is prevented by the transcription factors induced in response to endogenous CSF-1, thereby

antagonizing neutrophil specific transcription factors and blocking neutrophil development.

9.4 Future research

The work presented in this thesis was performed to characterize goldfish myeloid progenitor cells in terms of their surface receptors, growth factors, and transcription factors and examine their involvement in the process of macrophage and neutrophil cell development. These experiments provide a significant framework for the isolation and characterization of distinct myeloid progenitor cell subsets and will be essential for examining myeloid progenitor cell commitment and differentiation stages, akin to that of mammals. Although the work presented in this thesis represents a significant advance in characterizing myeloid progenitor cells, there are a number of future experiments that need to be performed to further our understanding of myeloid cell development and the progenitors that give rise to these differentiated cell types.

9.4.1 Generation of specific antibodies to the growth factor receptors as markers of progenitor cell subsets:

Now that I have identified and characterized goldfish *kita* and *gcsfr*, the generation of antibodies to the growth factor receptors KITA and GCSFR is extremely important for the identification and isolation of viable myeloid progenitor cells sub-populations. Currently, our methods consist of isolating progenitor cells using FACS sorting based on forward (size) and side (internal complexity) scatter properties. The resulting population comprises of a

heterogeneous mixture of HSCs, HPCs, lymphocytes, and possibly a small number of contaminating erythrocytes. The mixture of cell types and stages impedes our ability to definitively examine the effects of a defined, or combination of, growth factor(s) on the functional response of a particular myeloid progenitor cell stage. I propose the development of monoclonal antibodies for two reasons. (A) In most cases, monoclonal antibodies are used to identify HSC/HPC subsets in the mouse system as they recognize a single epitope on the target molecule and are less likely to cross-react with other similar epitopes on non-target molecules. This is key, as growth factor receptors are often expressed on the surface of a cell in low numbers (10-1000 receptors per cell), and even a small amount of cross-reactivity observed with a polyclonal antibody can cloud results and generate false positives. (B) Hybridoma cell lines can be maintained through serial passage or stored at -80^{o}C and thus represent an indefinite source of antibody. The monoclonal antibody would only need to be characterized once and would eliminate the batch to batch variability of polyclonal antibodies from different rabbits, either due to differences in protein preparation or in host response.

Using these monoclonal antibodies, I predict a number of progenitor cell subsets will be identified including KIT^{+}/CSF-1R^{-}/GCSFR^{-} (early HPCs), KIT^{+}/CSF-1R^{+} (committed macrophage progenitors) KIT^{-}/CSF-1R^{+} (monocyte precursors), KIT^{+}/GCSFR^{+} (committed granulocyte progenitors), and KIT^{-}/GCSFR^{+} (granulocyte precursors). These cell sub-populations would be useful in future experiments to determine their lineage potential, functional responses and the modulation of transcription factor expression in response to myeloid growth factors. Furthermore, I believe these monoclonal antibodies would be

instrumental in identifying a CSF-1R$^+$/GCSFR$^+$ subpopulation. This subpopulation would be an interesting discovery, if present, in the teleost system, as it may represent a GMP and suggest the combined signaling of CSF-1/CSF-1R and GCSF/GCSFR may act on this cell population and compensate for the loss of GM-CSF in teleosts. Although the production of monoclonal antibodies is a huge undertaking, their production is necessary to reliably identify progenitor cell subsets and for future experiments in isolating multipotent or lineage committed HPCs.

9.4.2 Kit/kit ligand system in teleosts.

In this thesis, I provided evidence for the role of KITA and KITLA in teleost myelopoiesis (Chapter 4), similar to that of mammalian SCF [48-50] [51, 52] and in contrast to that of the zebrafish model system [32, 33, 40]. The functional role of rgKITLA in the maintenance of progenitor cells is interesting and suggests that teleost KITLA may also play a role in maintaining HSPC quiescence or survival, as seen in mammalian systems. Therefore, I believe it would be worthwhile to determine if rgKITLA is capable of preventing progenitor cell apoptosis or in regulation of cell cycle, as mechanisms of KITLA signaling have not been reported in any teleost system.

Measurement of apoptosis in sorted progenitor cells in the presence and absence of rgKITLA can be measured in a number of ways [53]. However, I propose using a flow cytometry based propidium iodide (PI) DNA binding assay. Progenitor cells should be sorted and seeded into four wells with ± serum and ± rgKITLA. Various days post addition of rgKITLA should be assessed. Following

treatment of progenitors, cells can be fixed, permeabilized, incubated with PI and analyzed by flow cytometry. PI will bind to the cellular DNA and the fragmentation of DNA during apoptosis will result in a decrease in PI fluorescence away from the characteristic G_0/G_1 and small mitotic peak of DNA from viable cells. The shift in fluorescence can be quantified and related back to the level of apoptosis occurring. In addition, information on whether rgKITLA affects cell cycle regulation can be measured simultaneously as distinct peaks corresponding to the amount of DNA present in the cell will be detected.

Alternatively, improvements in the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay have modified it to be compatible with flow cytometry. In this assay, the ends of DNA fragments are labeled with bromolated deoxyuridine triphosphate nucleotides (BrdU) by the terminal deoxynucleotidyl transferase (TDT) enzyme and detected with fluorescently labeled antibodies. Cells with fragmented DNA will have increased BrdU incorporation, and will have a shift in fluorescence that can be quantified for comparison between samples.

Performing this experiment will provide information on the level of apoptosis that is occurring in the progenitor cell population within the PKM culture system in the absence of exogenous growth factors, and the level of apoptosis occurring in progenitor cells in the presence of rgKITLA. I predict that rgKITLA will block apoptosis of progenitor cells, leading to their survival. If rgKITLA is able to prevent apoptosis of progenitor cells and promote their survival *in vitro*, the continuous addition of rgKITLA to PKMs may prove useful in maintaining a long-term population of progenitor cells.

Additionally, if antibodies are generated to KITA, it would be interesting to identify populations of KITA$^+$ cells from tissues to assess their distribution, and the presence of KITA on cells of the myeloid lineage including progenitors, monocytes, macrophages and neutrophils (as described in Chapter 5). If KITA was expressed on monocytes, macrophages or neutrophils, the next step would be to investigate the role of KITA in phagocytosis of *A. salmonicida*. Cells could be incubated with the phagocyte in the presence or absence of cytochalasin D to block actin polymerization, important in formation of pseudopodia and the initiation of phagocytosis. Cells could then be incubated with the anti-KITA antibody followed by a fluorescently labeled secondary antibody and microscopically viewed to examine the distribution of KITA in these phagocytes compared to the normal distribution of KITA on cells not exposed to *A. salmonicida*. Whether *kita* is increased in phagocytes to simply increase survival signals or plays an active role in phagocytosis would provide novel information on the regulation of myeloid cell biology.

Currently it is yet unknown whether *kitb/kitlb,* orthologues of mouse *c-KIT/SCF,* were retained or lost during goldfish evolution. Since the mRNA distribution of zebrafish *kita* and *kitb* approximated the mRNA distribution of mouse *c-KIT* in tissues [32, 33], it stands to reason that the functions of mouse c-KIT may have been partitioned between teleost KITA and KITB. Therefore, I predict that teleost KITB/KITLB play an important role in teleost myelopoiesis that is different from that of KITA/KITLA. While I was unable to identify the goldfish *kitb/kitlb* orthologues using homology-based primers, I believe additional attempts should be made to identify *kitb/kitlb* in goldfish. Unfortunately, *kitb* or *kitlb* mRNA sequences have not been identified in other teleost species, precluding the

design of degenerate primers. However, the nucleotide sequence of the intracellular domain of *kitb* may be highly conserved in teleosts, and this region should be the target of primer design. Once identified, the molecular and functional characterization of *kitb* and *kitlb* should be performed in a similar manner to the experiments performed to characterize *kita* and *kitla,* (Chapter 4). Lastly, if *kitb/kitlb* genes can be identified in goldfish, it would be interesting to determine if KITLA and KITLB can bind to both KITA and KITB, or if they are restricted to binding their respective receptor. This experiment could be performed by a simple cross-linking experiment using recombinant proteins, as described in chapter 4 of this thesis. The identification and characterization of *kitla/kitla*, *kitb/kitlb* and their receptor-ligand interactions may shed light on the regulation of progenitor cells survival, proliferation and differentiation *in vivo* and *in vitro*.

9.4.3 Interaction of myeloid growth factors

While elucidation of the function of defined growth factors is an important step in understanding progenitor cell development, HPCs cells are exposed to complex mixtures of cytokines in the hematopoietic niche. Growth factors identified in the Belosevic laboratory include granulin [54], leukemia inhibitory factor (LIF) [55], CSF-1 [11, 12], and KITLA [34] (Chapter 4). Granulin and LIF appear to promote the proliferation of a population of cells within the progenitor cell gate, however, LIF only promoted cellular proliferation in the presence of PKM CCM, suggesting that LIF required a co-factor [55]. CSF-1 appeared to promote the proliferation of progenitor cells, but to a limited extent [12].

Therefore, the next step would be to elucidate the growth factor interactions that occur in terms of progenitor cell survival, proliferation, differentiation and regulation of transcription factors, (as described in Chapter 4 and 6). Based on the regulation of TF expression in progenitor cells when treated with KITLA and CSF-1, I would speculate that KITLA/CSF-1 and KITLA/LIF would synergize to promote progenitor cell to macrophage differentiation. However, it is difficult to speculate on the interaction of KITLA with granulin, as granulin appears to act on a population of progenitor cells, promoting their proliferation and generating a cellular population with slightly higher side scatter and the same forward scatter as the progenitor cell gate (unpublished observations). The identity of this cell population is unknown.

9.4.4 Goldfish granulocyte colony-stimulating factor

Now that I have identified and characterized a highly pure population of goldfish neutrophils as well as GCSFR (Chapters 7 and 8), the next logical step would be to identify the ligand(s), GCSF, in goldfish. Despite my continual efforts using homology based and degenerate primers on a number of non-stimulated and stimulated cell types, I was unable to identify goldfish *gcsf*. The identification of *gcsf* has been problematic in many teleost systems due to the high selection pressure that has been imposed on *gcsf*, leading to very low identity among teleosts (~27-33%) [37, 42, 43]. In the teleost species in which *gcsf* has been identified, transcripts were found using genomic resources or through mass screening of various libraries [37, 42, 43]. Therefore, in the absence of genomic information for the goldfish, I propose the isolation of goldfish PBLs, and the

subsequent stimulation of these cells with mitogens such as PMA/ConA/CaI for 6-12 hrs. Following the collection of RNA from non-treated and mitogen-treated cells, a subtractive cDNA library should be generated, and numerous clones sequenced in an attempt to identify *gcsf* transcripts. Alternatively, if GCSF cannot be identified by screening a cDNA library, recombinant GCSFR can be produced and coupled to beads to generate an affinity column in an attempt to isolate the native GCSF which can then be subjected to mass spectrometry to identified peptides from which degenerate primers can be designed against.

Once identified, recombinant GCSF can be functionally characterized in terms of its ability to mediate neutrophil differentiation and activation. I predict that kidney progenitor cells treated with GCSF will promote the proliferation and differentiation of progenitors into neutrophils, and may even give rise to some monocytes and macrophages, similar to that observed in zebrafish methylcellulose CFU assays [56]. These *in vitro* derived neutrophils should undergo morphological and cytochemical characterization similar to that described for primary kidney neutrophils (Chapter 7). Primary kidney neutrophils and *in vitro* derived neutrophils could be treated with GCSF and their functional responses (chemotaxis, ROI, NO, and degranulation, Chapter 7) compared. In addition, ligand-receptor interactions can be assessed through a cross-linking experiment to determine if GCSF forms homodimers, and interacts with two GCSFR molecules, as described for mammalian systems [57-59], or if teleost GCSF/GCSFR interaction is different from that of mammalian systems. I suspect the functional characterization of goldfish GCSF(s) will show an evolutionary conservation for the role of GCSF in neutrophil development. In addition, I believe GCSF(s) also possesses novel functions not observed for mammalian GCSF,

such as replacing GM-CSF, and may explain the high level of evolutionary pressure on GCSF in teleosts.

9.5 Summary

The use of the comparative teleost model system is expanding in the field of comparative immunology as a means of understanding the evolution of vertebrate myelopoiesis. Although vertebrate myelopoiesis appears to be highly conserved, based on the nugget of information we have accrued from bony fish, there are notable differences that warrant further study. Research has only just begun to scratch the surface of the soluble mediators, their receptors, and the intracellular machinery that govern teleost myelopoiesis.

Over the course of my thesis, I have cloned and characterized a number of hematopoietic growth factors, receptors, and transcription factors and assessed their role in myeloid progenitor cell biology. I believe my work has enhanced our understanding of the machinery involved in myelopoiesis of lower vertebrates.

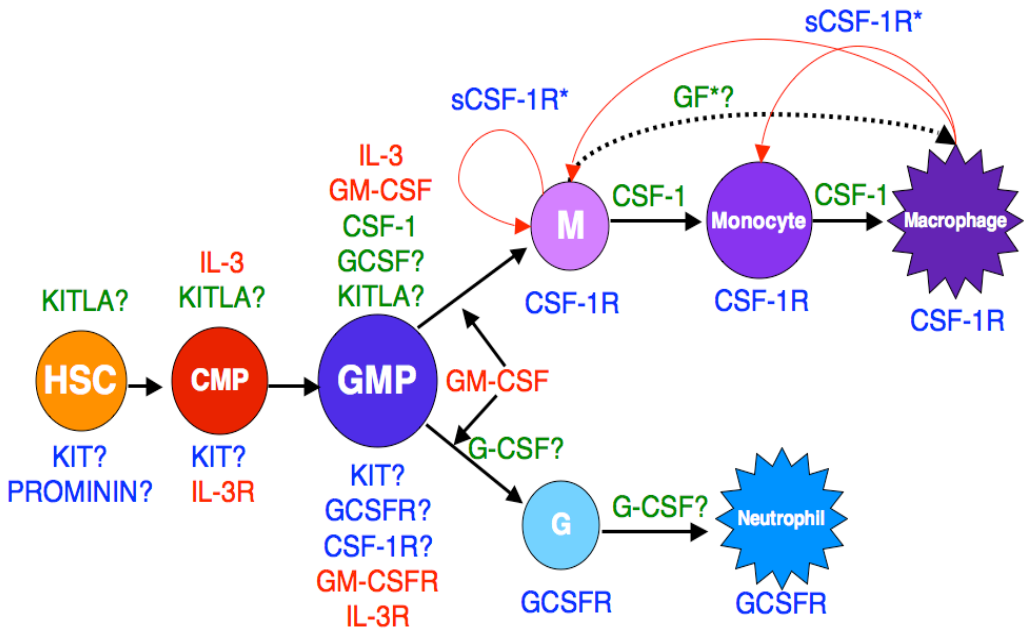


Figure 9.1: Growth factors and their receptors involved in goldfish myelopoiesis.
Goldfish growth factors are shown in green lettering, goldfish growth factor receptors/surface receptors are shown in blue lettering, and growth factors and their receptors important in mammalian myelopoiesis, but have yet to be identified in teleosts are shown in red lettering. The dashed arrow denotes the alternative pathway of macrophage development in goldfish, the red arrows denote negative regulation of macrophage development by sCSF-1R. Question marks denote the hypothesized role of growth factors or receptors and further studies are required to test the hypothesis. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1)**Cellular stages:** HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) **Growth factors:** KITLA, kit ligand a; IL-3, interleukin 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1 (macrophage colony-stimulating factor); GCSF, granulocyte colony-stimulating factor; GF, growth factor. **Receptors:** IL-3R, interleukin 3 receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; CSF-1R, colony-stimulating factor-1 receptor (macrophage colony-stimulating factor receptor); sCSF-1R, soluble colony-stimulating factor-1 receptor; GCSFR, granulocyte colony-stimulating factor receptor.

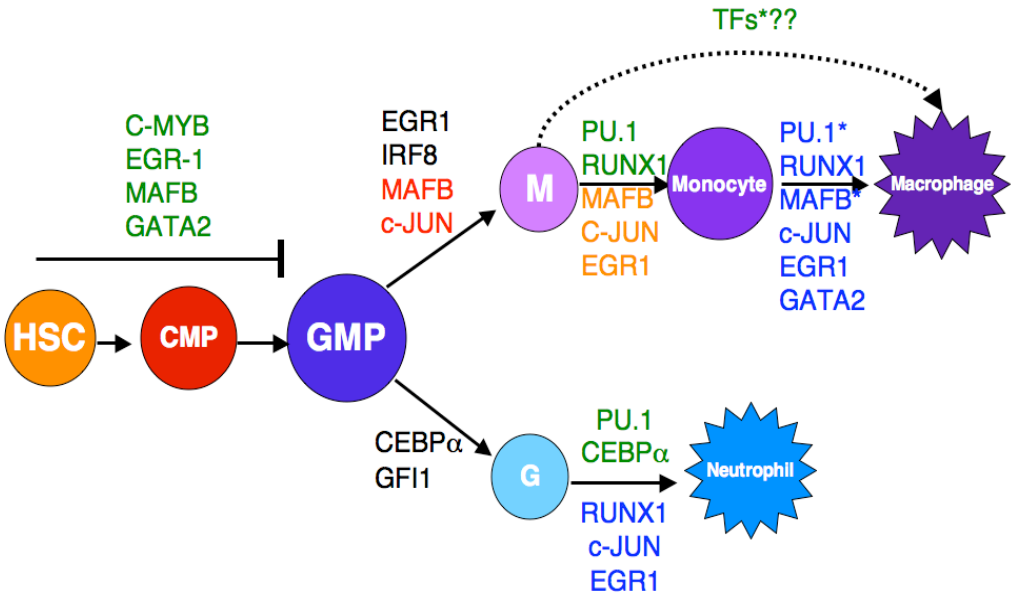


Figure 9.2: Transcription factors involved in goldfish myelopoiesis. Goldfish transcription factors shown in green lettering are up-regulated, goldfish transcription factors that are important in cellular differentiation in mammalian systems but show no change in the goldfish system are shown in orange lettering, goldfish transcription factors shown in blue lettering are down-regulated, transcription factors shown in black lettering are known to be involved in teleost systems but have yet to be studied in the goldfish system, finally, transcription factors important in cellular junctions in the mammalian system but have yet to be studied in the teleost system are shown in red lettering. The dashed arrow denotes the alternative pathway of macrophage development in teleosts. Question marks denote unknown transcription factors involved in the alternative pathway of macrophage development. Asterisks mark differences between teleosts and mammals. Abbreviations used: (1) **Cellular stages:** HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; M, monocytic precursor; G, granulocytic precursor. (2) **Transcription factors:** c-MYB, cellular myelobastosis oncogene; EGR-1, early growth response-1; MAFB, musculoaponeurotic fibrosarcoma oncogene homologue B; GATA2, GATA binding protein 2; IRF8, interferon regulatory factor 8; CEBPα, CCAAT/enhancer-binding protein alpha; GFI1, growth factor independent 1; RUNX1, runt-related transcription factor 1.

9.6 References

[1] Bertrand JY, Kim AD, Teng S, Traver D. CD41^{+} cmyb^{+} precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. Development. 2008;135(10):1853-62.

[**2] Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI.** Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. Nat Immunol. 2003;4(12):1238-46.

[3] Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, et al. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004;104(5):1298-305.

[4] Kobayashi I, Saito K, Moritomo T, Araki K, Takizawa F, Nakanishi T. Characterization and localization of side population (SP) cells in zebrafish kidney hematopoietic tissue. Blood. 2008;111(3):1131-7.

[5] Kobayashi I, Sekiya M, Moritomo T, Ototake M, Nakanishi T. Demonstration of hematopoietic stem cells in ginbuna carp (*Carassius auratus langsdorfii*) kidney. Dev Comp Immunol. 2006;30(11):1034-46.

[6] Kobayashi I, Moritomo T, Ototake M, Nakanishi T. Isolation of side population cells from ginbuna carp (*Carassius auratus langsdorfii*) kidney hematopoietic tissues. Dev Comp Immunol. 2007;31(7):696-707.

[7] Moritomo T, Asakura N, Sekiya M, Ototake M, Inoue Y, Nakanishi T. Cell culture of clonal ginbuna crucian carp hematopoietic cells: differentiation of cultured cells into erythrocytes *in vivo*. Dev Comp Immunol. 2004;28(9):863-9.

[8] Kobayashi I, Kuniyoshi S, Saito K, Moritomo T, Takahashi T, Nakanishi T. Long-term hematopoietic reconstitution by transplantation of kidney hematopoietic stem cells in lethally irradiated clonal ginbuna crucian carp (*Carassius auratus langsdorfii*). Dev Comp Immunol. 2008;32(8):957-65.

[9] Neumann NF, Barreda D, Belosevic M. Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Dev Comp Immunol. 1998;22(4):417-32.

[10] Neumann NF, Barreda DR, Belosevic M. Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus* L.) kidney leukocyte cultures. Fish Shellfish Immunol. 2000;10(1):1-20.

[11] Hanington PC, Hitchen SJ, Beamish LA, Belosevic M. Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. Fish Shellfish Immunol. 2009;26(1):1-9.

[12] Hanington PC, Wang T, Secombes CJ, Belosevic M. Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony-stimulating factor-1. J Biol Chem. 2007;282(44):31865-72.

[13] Barreda DR, Hanington PC, Belosevic M. Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol. 2004;28(5):509-54.

[14] Guilbert LJ, Stanley ER. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol. 1980;85(1):153-9.

[15] Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell. 1985;41(3):665-76.

[16] Roca FJ, Sepulcre MA, Lopez-Castejon G, Meseguer J, Mulero V. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. Mol Immunol. 2006;43(9):1418-23.

[17] Mulero I, Pilar Sepulcre M, Roca FJ, Meseguer J, Garcia-Ayala A, Mulero V. Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. Dev Comp Immunol. 2008;32(10):1151-9.

[18] Barreda DR, Hanington PC, Stafford JL, Belosevic M. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2005;29(10):879-94.

[19] Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404(6774):193-7.

[20] Dzik JM. The ancestry and cumulative evolution of immune reactions. Acta Biochim Pol. 2010;57(4):443-66.

[21] Tauber AI. Metchnikoff and the phagocytosis theory. Nat Rev Mol Cell Biol. 2003;4(11):897-901.

[22] Muller WE, Muller IM. Origin of the metazoan immune system: identification of the molecules and their functions in sponges. Integr Comp Biol. 2003;43(2):281-92.

[23] Smith LC, Rast JP, Brockton V, Terwilliger DP, Nair SV, Buckley KM, et al. The sea urchin immune system. Information Systems Journal. 2006;325-39.

[24] Lin X, Soderhall I. Crustacean hematopoiesis and the astakine cytokines. Blood. 2011;117(24):6417-24.

[25] Crozatier M, Vincent A. Drosophila: a model for studying genetic and molecular aspects of haematopoiesis and associated leukaemias. Dis Model Mech. 2011;4(4):439-45.

[26] Crozatier M, Meister M. Drosophila haematopoiesis. Cell Microbiol. 2007;9(5):1117-26.

[27] Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen AH, Terwilliger DP, et al. The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol. 2006;300(1):349-65.

[28] Nobrega MA, Pennacchio LA. Comparative genomic analysis as a tool for biological discovery. J Physiol. 2004;554(Pt 1):31-9.

[29] How GF, Venkatesh B, Brenner S. Conserved linkage between the puffer fish (*Fugu rubripes*) and human genes for platelet-derived growth factor receptor and macrophage colony-stimulating factor receptor. Genome Res. 1996;6(12):1185-91.

[30] Williams H, Brenner S, Venkatesh B. Identification and analysis of additional copies of the platelet-derived growth factor receptor and colony stimulating factor 1 receptor genes in fugu. Gene. 2002;295(2):255-64.

[31] Williams H, Brenner S, Venkatesh B. Characterization of the platelet-derived growth factor receptor alpha and c-kit genes in the pufferfish Fugu rubripes. DNA Seq. 2002;13(5):263-70.

[32] Parichy DM, Rawls JF, Pratt SJ, Whitfield TT, Johnson SL. Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. Development. 1999;126(15):3425-36.

[33] Mellgren EM, Johnson SL. kitb, a second zebrafish ortholog of mouse Kit. Dev Genes Evol. 2005;215(9):470-77.

[34] Katzenback BA, Belosevic M. Molecular and functional characterization of kita and kitla of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2009;33(11):1165-75.

[35] Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. Dev Biol. 2001;238(2):274-88.

[36] Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, et al. Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. Mol Immunol. 2005;42(1):1-8.

[37] Liongue C, Hall CJ, O'Connell BA, Crosier P, Ward AC. Zebrafish granulocyte colony-stimulating factor receptor signaling promotes myelopoiesis and myeloid cell migration. Blood. 2009;113(11):2535-46.

[38] Katzenback BA, Belosevic M. Characterization of granulocyte colony stimulating factor receptor of the goldfish (*Carassius auratus* L.). Dev Comp Immunol. 2012;36(1):199-207.

[39] Liu L, Korzh V, Balasubramaniyan NV, Ekker M, Ge R. Platelet-derived growth factor A (pdgf-a) expression during zebrafish embryonic development. Dev Genes Evol. 2002;212(6):298-301.

[40] Hultman KA, Bahary N, Zon LI, Johnson SL. Gene Duplication of the zebrafish kit ligand and partitioning of melanocyte development functions to kit ligand a. PLoS Genet. 2007;3(1):e17.

[41] Wang T, Hanington PC, Belosevic M, Secombes CJ. Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. J Immunol. 2008;181(5):3310-22.

[42] Santos MD, Yasuike M, Hirono I, Aoki T. The granulocyte colony-stimulating factors (CSF3s) of fish and chicken. Immunogenetics. 2006;58(5-6):422-32.

[43] Nam BH, An GH, Baeck GW, Kim MC, Kim JW, Park HJ, et al. Molecular cloning and expression of cDNAs for two distinct granulocyte colony stimulating factor genes from black rockfish *Sebastes schlegelii*. Fish Shellfish Immunol. 2009;27(2):360-4.

[44] Davidson AJ, Zon LI. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. Oncogene. 2004;23(43):7233-46.

[45] de Jong JL, Zon LI. Use of the zebrafish system to study primitive and definitive hematopoiesis. Annu Rev Genet. 2005;39481-501.

[46] Taylor JS, Van de Peer Y, Braasch I, Meyer A. Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond B Biol Sci. 2001;356(1414):1661-79.

[47] Ravi V, Venkatesh B. Rapidly evolving fish genomes and teleost diversity. Curr Opin Genet Dev. 2008;18(6):544-50.

[48] Ogawa M, Matsuzaki Y, Nishikawa S, Hayashi S, Kunisada T, Sudo T, et al. Expression and function of c-kit in hemopoietic progenitor cells. J Exp Med. 1991;174(1):63-71.

[49] Keller JR, Ortiz M, Ruscetti FW. Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. Blood. 1995;86(5):1757-64.

[50] Thoren LA, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, et al. Kit regulates maintenance of quiescent hematopoietic stem cells. J Immunol. 2008;180(4):2045-53.

[51] Broudy VC. Stem cell factor and hematopoiesis. Blood. 1997;90(4):1345-64.

[52] Okumura N, Tsuji K, Ebihara Y, Tanaka I, Sawai N, Koike K, et al. Chemotactic and chemokinetic activities of stem cell factor on murine hematopoietic progenitor cells. Blood. 1996;87(10):4100-8.

[53] Sgonc R, Gruber J. Apoptosis detection: an overview. Exp Gerontol. 1998;33(6):525-33.

[54] Hanington PC, Barreda DR, Belosevic M. A novel hematopoietic granulin induces proliferation of goldfish (*Carassius auratus* L.) macrophages. J Biol Chem. 2006;281(15):9963-70.

[55] Hanington PC, Belosevic M. Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. Dev Comp Immunol. 2007;31(8):817-29.

[56] Stachura DL, Svoboda O, Lau RP, Balla KM, Zon LI, Bartunek P, et al. Clonal analysis of hematopoietic progenitor cells in the zebrafish. Blood. 2011;118(5):1274-82.

[57] Aritomi M, Kunishima N, Okamoto T, Kuroki R, Ota Y, Morikawa K. Atomic structure of the GCSF-receptor complex showing a new cytokine-receptor recognition scheme. Nature. 1999;401(6754):713-7.

[58] Tamada T, Honjo E, Maeda Y, Okamoto T, Ishibashi M, Tokunaga M, et al. Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. Proc Natl Acad Sci U S A. 2006;103(9):3135-40.

[59] Honjo E, Tamada T, Maeda Y, Koshiba T, Matsukura Y, Okamoto T, et al. Crystallization of a 2:2 complex of granulocyte-colony stimulating factor (GCSF) with the ligand-binding region of the GCSF receptor. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2005;61(Pt 8):788-90.