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SOLUBLE NEGATIVE REGULATORS OF GOLDFISH PRIMARY KIDNEY MACROPHAGE DEVELOPMENT

by

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I learned something: nobody knows everything.

This thesis is dedicated to all the people who struggle for human rights.

ABSTRACT

The generation of macrophages requires the coordinated responses to stimulatory and inhibitory signals that cell receive from their environment. While the up-regulation of macrophage production and survival is essential to fortify the immune system, their down-regulation is also vital to prevent macrophage related diseases and malignancy. Previous studies in goldfish showed that primary kidney macrophages release endogenous growth inducing factors into proliferative phase supernatant, which up-regulate their proliferation and survival. In this thesis, the effect of senescence phase supernatant (collected from goldfish primary kidney macrophage cultures) on goldfish primary kidney macrophages proliferation, survival and its impact on the ability of macrophages to tolerate H_2O_2 was analyzed. The results showed that the senescence phase supernatant down-regulated the proliferation and survival, and decreased the chemical tolerance of the cells. This indicated that the goldfish primary kidney macrophages promote targeted control of their proliferation and survival by secreting endogenous growth inhibitory factors in the senescence phase supernatant.

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LIST OF ABBREVIATIONS

7AAD:	7Aminoactinomycine D
APC:	Antigen presenting cell
BCA:	Bicinchoninic Acid
BrdU:	BromodeoxyUridine
CCM:	Cell conditioned medium
CD:	Cluster of differentiation
CSF-1:	Colnoy stimulating factor-1
CSF-1R:	Colony stimulating factor-1 receptor
ELISA:	Enzyme linked immunosorbant assay
EPo:	Erythropoietin
FACS:	Fluorescence Activated cell sorting
FMS:	Feline sarcoma virus
FSC:	Forward scatters
G-CSF:	Granulocyte colony stimulating factor
G-CSFR:	Granulocyte colony stimulating factor receptor
GM-CSF:	Granulocyte macrophage colony stimulating factor
GM-CSFR:	Granulocyte macrophage colony stimulating factor-receptor
IL:	Interleukin
IFN-gamma:	Interferon gamma
LPS:	lipopolysaccharide
M-CSF:	Macrophage colony stimulating factor
MAF:	Macrophage activating factors
MHCI:	Major histocompatibility class I
MHCII:	Major histocompatibility class II
MPS:	Mononuclear phagocytic system
PAMP:	Pathogen associated molecular pattern
PBS:	Phosphate buffered saline
PCD	Programmed cell death
PKM:	Primary kidney macrophages

PS:	Phosphotydilsyrine
PRR:	Pattern recognition receptor
PI3K:	Phosphatidylinositol 3 kinase
RNA:	Ribonucleic acid
NK:	Natural killer
NKT:	Natural killer T cell
ROI:	Reactive oxygen intermediates
rsCSF-1R:	Recombinant soluble colony stimulating factor-1 receptor
SDS-PAGE:	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
SH2:	src homology 2
SSC:	Side scatters
STAT:	Signal transducer and activator of transcription
TNF:	Tumor necrosis factor
TLR:	Toll like receptor

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SOLUBLE NEGATIVE REGULATORS OF GOLDFISH PRIMARY KIDNEY MACROPHAGE DEVELOPMENT

CHAPTER 1: INTRODUCTION AND REVIEW

General introduction

The immune system is a network of cells, tissues, and organs that work together to defend the body against foreign invaders and cancer cells. It identifies self from non-self, as well as abnormal self-cells like those derived from cancers. It fights and removes pathogens such as bacteria, parasites, fungi and virus as well as tumor cells that can cause disease (1). Broadly, the immune system can be divided into innate and the adaptive arms of immunity. These are not mutually exclusive and can overlap in different ways in their function. In general the immune system consists of organs, tissues various sites that harbor immune cells and different classes of immune cells.

Macrophages are a heterogeneous group of immune cells that contribute to a wide range of biological activities associated with host defenses and homeostasis. In relation to host defense, macrophages are involved in both innate and adaptive immune responses. They link the innate and adaptive immune system by virtue of their multifunctional role in responses such as antigen presentation, Fc receptor and complement-dependent phagocytosis, and inflammatory responses. They also play key roles in the induction of adaptive immune responses through the secretion of specific cytokines (2). In addition to the immune system, macrophages are also important in body homeostasis through

the removal of spent cells and debris in the course of tissue turnover during basal and inflammatory processes. Macrophages also play a central role in development of the nervous system, pancreas, and male and female fertility (3). They produce cytokines and growth factors, which regulate the proliferation and survival of their own, and other immune cells. As the result of these multiple functions, understanding the macrophage biology is a key aspect for understanding of host defense and homeostasis. Therefore, the study of regulatory mechanisms of their production and functions has an indispensable contribution to the advancement of scientific knowledge and clinical trials for the prevention or treatments of macrophages associated diseases and malignancy.

A central regulator of macrophage proliferation, differentiation, and survival is the macrophage colony-stimulating factor (M-CSF or CSF-1) (4). The action of CSF-1 is mediated by its membrane bound receptor, the CSF-1R. Understanding the regulatory mechanisms for macrophage is important for up regulation of macrophage production and function for defense against infection. Control of their numbers, stage of differentiation and effecter functions is also critical for the maintenance of homeostasis and may serve to elucidate therapeutic targets for macrophage-related chronic inflammation and malignant diseases. Since their original discovery by Elie Metchnikoff, significant work has been done to understand the macrophage biology and their role in immune system especially in higher vertebrates including mice and humans (5) Because of their complex biology and their contribution in host defense and homeostasis, further investigation of macrophage biology is important. In contrast to mammalian

systems, our understanding of macrophage biology in lower vertebrates is still in its infancy. Among others, this is important to trace the evolutionary development of the immune system from lower to higher vertebrates, and to expand our existing knowledge of potential applications in therapeutics for higher vertebrates.

Understanding the fish immune system is of considerable interest in aquaculture and fisheries. It is vital for fish disease diagnosis, treatment and development of effective vaccines. Fish also serve as a model for studying the immune system in higher vertebrates and as a bioindicators in environmental pollution studies. Recent studies in our group have established a framework for study of macrophage biology in lower vertebrates (6). Among others, techniques have been established for establishment of goldfish primary kidney macrophage (PKM) *in vitro* cultures. As the continuation of these studies my thesis work focuses on the negative regulation of goldfish primary kidney macrophage development *in vitro*.

1.2. Thesis objectives

Goldfish primary kidney macrophage cultures pass through different growth phases during *in vitro* development: these include a lag phase, a proliferative phase and a senescence phases (19). The shift from the proliferative phase to the senescence phase marks the transition between periods for active production of macrophage endogenous growth factors to one where macrophage growth inducing activity diminishes significantly. Cell conditioned media (CCM) collected from proliferative phase PKM cultures contain growth-inducing factors that further induce cell proliferation *in vitro*. On the other hand CCM collected

from senescence phase PKM culture is poor inducer of cell proliferation. Instead, it negatively impacts cell proliferation. (The term "supernatant" refers to "CCM" and used interchangeably throughout the thesis). Interestingly, our group recently identified a novel soluble form of the CSF-1 receptor. We believe that this represents one of potentially several negative inducers of macrophage development that are actively produced during the senescence phase of PKM development. The presence of the soluble form of CSF-1R in the senescence phase PKM culture supernatant and the inhibition of cell proliferation by recombinant soluble form of CSF-1R suggests that goldfish PKM cells are self regulating cells capable of producing growth inhibitory factors to the senescence phase supernatant, which negatively regulate the proliferation and survival of the PKM cultures.

The overall objective of my MSc thesis was to determine the impact of PKM senescence phase conditioned media on PKM proliferation, survival, and transition through previously defined phases of goldfish macrophage *in vitro* development. Our long-term objective is to understand the role of soluble form of CSF-1R and potentially other growth inhibitory factors contained in senescence phase supernatants in goldfish PKM endogenous control of macrophage development and function.

In chapter 1 of this thesis I review the background from the previous work done on macrophages and the role of CSF-1 in macrophage production and function. A second goal is to provide a literature review that highlights the present status of this area of research. Chapter 2 contains a description of the materials

and methods used during my thesis work. In Chapter 3, I discuss the *in vitro* generation of goldfish primary macrophage cells. I expand on previously characterized phases of *in vitro* goldfish PKM development using flow cytometric, microscopic and ImageStream-based analysis. In Chapter 4, I describe the effect of senescence phase supernatant on goldfish primary kidney macrophage cell proliferation and survival. I report that senescence phase supernatants contribute to the shift from the proliferative phase PKM to senescence, and its effect on the survival of goldfish PKM cells by induction of early cell death in the proliferative phase goldfish PKM cells. I also describe how it affects the goldfish PKM cells chemical tolerance under oxidative stress conditions, and the effect of the senescence phase supernatant on the goldfish PKM cell proliferation. I then focus on the production of recombinant goldfish soluble colony stimulating factor-1 receptor using an insect expression system. Chapter 5 contains the general discussion from my findings with respect to my objectives and explores their relevance in view of the available literature. The goal is to offer some direction to future research endeavors examining the role of soluble form of CSF-1R in macrophage biology and its application as a means of therapeutics for malignancy and inflammation.

1.3. LITERATURE REVIEW

1.3.1. Macrophages

Macrophages are a heterogeneous cell population displaying different morphology and functions based on their locations in the body. There are two theories about the origin of macrophages. The reticuloendothelial system (RES), described by Aschoff in 1924 (7) is based on the location of the cells and vital staining. According to this theory macrophages are grouped with reticular connective tissues (from vascular blood or lymph vessels) and histocytes based on their phagocytic properties. The second theory is the mononuclear phagocyte system (MPS), suggested to replace the RES. It proposed by Van Furth (8) and includes tissue macrophages, circulating monocytes and promonocytes. The MPS theory grouped macrophages based on their origin (5), which assumes all the macrophages are derived from monocytes, are terminally differentiated, non-dividing, and short living cells. More recently, investigators have found that macrophages have a potential to proliferate and some macrophages can live for an extended periods (9). As any other type of blood cells, macrophages are also produced through the process of hematopoiesis.

1.3.2. Hematopoiesis

Hematopoiesis is the process of the formation and development of the blood cells. Development of the hematopoietic system is a complex process that occurs in various hematopoietic organs in different species and different developmental stages of the same species. The initial hematopoietic activity

appears in the blood island of the yolk sac during embryonic development. Adult-type definitive hematopoiesis begins in the aorta-gonad-mesonephros (AGM) region and thereafter shifts to the fetal liver where production of various hematopoietic cells occurs. Subsequently, hematopoiesis shifts to the bone marrow and spleen. In case of fish, the kidney represents the main hematopoietic organ (10). The bone marrow stem cells first differentiate into myeloid precursor cells. The myeloid precursor cells are intermediate cells that can follow different paths under the influence of cytokines including IL-3, IL-6, IL-7, GM-CSF, G-CSF, CSF-1 and EPO (11). These cytokines represent environmental stimuli that promote the differentiation of the myeloid precursors into different phagocytes including polymorphonuclear granulocytes (neutrophils) and mononuclear phagocytes (monocytes and macrophages).

During hematopoietic cell development multipotential stem cells located in hematopoietic organs (yolk sac, fetal liver, spleen, kidney and bone marrow), self-renew and give rise to committed progenitor cells. These progenitor cells ultimately develop into different families of mature hematopoietic cells, under the influence of lineage specific growth factors. Since the life span of the mature hematopoietic cells is relatively short, the stem cells and growth factors provide a mechanism for continuous flow of differentiating cells (12). Multipotent stem cells give rise to committed progenitors, which pass through monoblast, promonocyte, and monocyte stages. The latter enters the circulatory system in route to the various tissues (13). There in response to local signals monocytes differentiate to mature tissue macrophages. The differentiation of macrophage

progenitors to mature macrophages encompasses morphological, biochemical, and functional changes (14).

1.3.3. Macrophage proliferation

Colony-stimulating factors are important cytokines that contribute to the proliferation of specific progenitor cells to form mature neutrophilic granulocytes and macrophages (15). These cytokines include colony stimulating factor -1(CSF-1), granulocyte colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF). They are hematopoietic growth factors and regulate the production of blood cells. Of these cytokines, the colony stimulating factor-1 (CSF-1) and granulocyte-macrophage colony stimulating factors (GM-CSF) play key roles in the production of macrophages from the hematopoietic progenitor cells. CSF-1 is the main cytokine for proliferation, differentiation and survival of monocyte, macrophages and their progenitor cells. IL-3 also plays a crucial role in regulation of macrophage proliferation (16). For example, studies in murine macrophages (17) show that CSF-1, GM-CSF and IL-3 activate Na-K ATPase and induce DNA synthesis, which is important for macrophage proliferation. Once inside tissue or inflammatory site the number of macrophages can also be maintained through proliferation (18).

1.3.4. Macrophage differentiation

Colony stimulating factors also play a key role in the differentiation along the macrophage lineage (14). As macrophages are pivotal cells in mediating cellular and humoral immune mechanisms, their continuous renewal and differentiation into functionally mature effecter cells is vital to host defense. In

addition to proliferation and self-renewal, the expired populations of tissue macrophages maintain their numbers by infiltration and differentiation of circulating monocytes (19).

1.3.5. Cytokines in macrophage proliferation and differentiation

Cytokines are pleiotropic, redundant and multifunctional in nature; different cytokines have a similar effect (redundant) or single cytokines may have several functions (pleiotropic). As the result, complex networks of cytokines contribute to the production and activation of macrophages by acting on macrophages or their progenitors. As described above, the colony-stimulating factors are well-studied and they play a key role in hematopoiesis that are important in the proliferation, differentiation, survival and function of macrophages and their progenitor cells at different developmental stages. My review will focus on GM-CSF and CSF-1 as representative central modulators of macrophage development and function (20-21).

1.3.5.1. Granulocyte macrophage -colony stimulating factor (GM-CSF)

The granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of the hematopoietic growth factors that can stimulate the proliferation, maturation and function of hematopoietic cells. It has profound effects on the functional activities of various circulating leukocytes including lymphocytes, monocytes, and neutrophils, to enhance their functions in host defense (22). Various types of cells like T cells, macrophages, endothelial cells and fibroblasts cells produce the GM-CSF (23). The cytokines, interleukin-3 (IL-3), and

interleukin-5 (IL-5) are also growth factors that have overlapping activities in the regulation of hematopoietic cells with the GM-CSF (24).

1.3.5.2. Granulocyte macrophage -colony stimulating factor receptor (GM-CSFR)

The granulocyte macrophage colony-stimulating factor receptor is essential in the production of white blood cells, augments of their functions, and inhibition of apoptosis in myeloid cells. It is a heterodimer receptor and contains an extracellular domain, a transmembrane domain and a cytoplasmic domain (25). This receptor composed of at least two different subunits, an α chain, which is specific to the GM-CSF, and a β chain, which also shared by IL-3 and IL-5 (26). The alpha subunit binds the ligand while the beta subunit is for signal transduction. Both the alpha and the beta subunits are members of the cytokine receptor superfamily (27).

1.3.5.3. Signal transduction by GM-CSF and its receptor

Receptors for GM-CSF, IL-3 and IL-5 share common β sub unit that is responsible for the signal transduction. The β subunit does not bind to the cytokine by itself but forms a high-affinity receptor of α subunit. Both α and β sub units have a typical motif of the cytokine receptor super family in their extracellular domain (28). Binding of the cytokine to its receptor causes the dimerisation of the α and β subunits. Subsequently the tyrosine residues of β subunit are phosphorylated by members of the Janus kinase (JAK) family (29). This leads to the association with an Shc adaptor protein. Then Shc interacts with

GRB2/SoS complex and results in activation of more molecules in the pathway, downstream, and result, in the cellular response.

1.3.5.4. Colony stimulating factor -1(CSF-1)

Macrophage activation is a key determinant of susceptibility and pathology in a variety of inflammatory diseases. The extent of macrophage activation is tightly regulated by a number of pro-inflammatory) and anti-inflammatory cytokines. Macrophage colony stimulating has a proinflammatory role in addition to differentiation, growth and survival factor for monocytes/macrophages and their bone marrow progenitors (7,12, 30,31). It has also been demonstrated to play important roles in bone metabolism, atherogenesis, and pregnancy and development of the female reproductive tract (32). The CSF-1 has three isoforms, which is encoded in different mechanisms by the same gene. All the three isoforms have a common N-terminal sequence, which is responsible for the biological activity of the cytokine (including the 32 aa peptide signal) and a common C-terminal sequence including the transmembrane domain. The mature CSF-1 isoforms probably differ in their production sites and physiological functions (33). In general little is known about the CSF-1 biology in lower vertebrates (34) and need further studies to understand its production and function.

1.3.5.5. Colony stimulating factor-1 receptor (CSF-1R)

The CSF-1R is a member of the type III receptor tyrosine kinase (RTK) family, which includes c-Kit, platelet-derived growth factor receptor α and β, and Flt3 (1). It contains an extracellular domain of five Ig-like domains, a

transmembrane domain, and an intracellular tyrosine kinase domain. All of the functions of Colony stimulating factor-1 (CSF-1) are mediated by CSF-1R, which is the primary regulator of the common myeloid lineage (35). Binding of CSF-1 to this receptor on cells results in tyrosine phosphorylation of the receptor and many other proteins that can activate downstream signaling pathways (36).

1.3.5.6. Signal transduction by CSF-1 and its receptor

The extracellular domain of CSF-1R has five clusters of immunoglobulin like domains. The first three domains have high affinity for CSF-1. When the cytokine binds to its receptor it results in receptor dimerization, autophosphorylation, and activation, of its cytoplasmic tyrosine residues. These are then used as docking sites for SH2-containing signaling proteins. There are at least five main tyrosine autophosphorylation sites in this cytoplasmic domain leading to the activation of Ras-ERK1/2 and class IA PI3K and to the formation of DNA-binding complexes containing various STAT families. CSF-1R also recruits Src family kinases (SFK) via an autophosphorylation site in the juxtamembrane domain. The subsequent interactions of phosphorelated cytoplasmic domain with primary adaptor protein result in initiation of signal along the specific pathways (37), which finally produce overall cellular response (32,38).

1.3.6. Soluble regulators of macrophage production and function

Cytokines are low molecular weight proteins that are central to the regulation of immune cell survival, development, activation, and function (39). The generation of macrophages requires the coordinated response to excessive

stimulatory and inhibitory signals that cells receive from their environment. This is directly related to the molecule and their receptors that induce the signals. The consequences of the cytokines and their receptor over-production are serious in hematopoietic and immune responses. Similarly, low production or their complete absence also has biological effects (40). As the result, regulation of their production both in magnitude and duration is important for the control of target cells (41). There are different factors that positively or negatively regulate the macrophage production and functions. This thesis focuses on the negative regulators.

Various factors might impact negatively and have an inhibitory effect on the macrophage production and functions both *in vitro* and *in vivo*. Among others, environmental factors such as physical cell-to-cell contact, oxidative stress, toxic chemicals from metabolic products or pathogens, and nutrient depletion play a significant role (42, 43). Others such as inhibition of the production and function of cytokines that act on the macrophages (inhibition of the production of CSF-1, GM-CSF, or IL-3 and their respective receptors) could also negatively affect the production and function of macrophages. For instance, some negative regulators exert their effect in different mechanisms on cytokine production and functions like by regulating the gene expression for both cytokine and its receptors through changing the gene transcription, mRNA stability, and by translational and post translational modifications. Some still regulate macrophage production and function by inhibiting the binding of cytokine to its receptor, or by inhibiting downstream signal cascades. Among others, the inhibition of binding a cytokine

to its receptor can be achieved by soluble proteins that bind to the receptors, or by alternative soluble receptors, which competes to their membrane-bound receptor counterparts. These soluble forms of receptors keep the cytokine away from the membrane-bound receptors. Moreover, macrophage production and function can be negatively impacted by other cytokine activity (41) such as tumor necrosis factor-alpha (TNF alpha), interferon-gamma (IFN gamma), or lipopolysaccharide (LPS) (18). In general, in the regulation of macrophage production and proliferation, regulating the colony- stimulating factors play a crucial role at different developmental stage of the cells (43).

1.3.7. Negative regulation of macrophages production and function and its relevance to immunity

A common theme in immune regulation takes advantage of the balance of positive (activatory) and negative (inhibitory) signals. Failure in the inhibitory stimulus may cause chronic inflammatory diseases such as the inflammatory bowel diseases (IBD) (44), macrophage activation syndrome (MAS) (45), autoimmune diseases or cancers. Inappropriate inhibition has also its own problems. For example, during infection some pathogens have mechanisms to downregulate the macrophage production and activation as their survival mechanisms. For example, *Leshimania* and *Ehrlichia chaffeensis* survives inside the macrophage by inhibiting the production of proinflammatory cytokines (46). Studies in higher vertebrates (mice and human) show that there are different molecules or factors that negatively regulates the macrophage production and function in immune responses. Among these, BCL-6 (47), IL-10 (48), activation of cAMP-dependent protein kinase (49), Adenosine, (50), Decorine, (51)

negatively affect the macrophage production and function either by inhibiting the production of proinflammatory cytokines or inhibiting DNA synthesis during macrophage proliferation.

1.3.8. Negative regulation of macrophages production and function and its relevance in homeostasis

Macrophages also play a key role in body homeostasis. They are crucial in clearing debris during inflammatory reactions, tissue turnover or embryonic development. They also release cytokine and growth factors during tissue remodeling, wound healing and tissue repair process (52). Macrophages in different tissues play key roles in normal physiology and homeostasis such as bone resorption and normal skeletal development (53), in response of the liver to toxic compounds, (54), and in the development of the endocrine system (55). In general, macrophages are important in morphogenesis, angiogenesis, bone development, the generation of adipose tissue and neuronal patterning. In each case, excess or depletion of macrophage production and activation affects the formation of the tissue and compromises its function (56); hence, proper regulation of macrophage production and function is crucial homeostasis and normal development (57).

1.3.9. Over-expression of CSF-1 or its membrane bound receptor and its consequences

As described above, CSF-1 controls the survival, proliferation and differentiation of monocyte-macrophage lineage both *in vitro* and *in vivo* and these functions are mediated by its membrane bound receptor. There are some

common disorders related to over expression of the CSF-1 or its receptor, such as lupus nephritis (in kidney) (58), cancers of the reproductive system (breast, endometrium, ovarian, prostate) (59), in the regulation of microglia inflammatory responses (60), and hepatoma (61). The study of CSF-1 and its receptor (both membrane bound and soluble form); therefore, is important not only for understanding the temporal, molecular and structural features that determine and regulates hematopoietic proliferation and differentiation, but also crucial to understand leukomogenesis and its treatment (62).

1.3.10. Soluble receptors

Many membrane-bound receptors also exist as secreted soluble forms. The possible physiological roles of soluble cytokine receptors may include: (1) transport the cytokines. (2) Prevention of the cytokine degradation (3). Inhibition of the cytokine action by competing to their membrane bound receptor or (4) to reduce the cell responsiveness to the cytokine by shading membrane bound receptors. Therefore, soluble receptor for the cytokines appears to play key roles in the inhibition of the cytokine actions so that negatively regulates the production and activation of target cells (39, 63, 64).

Soluble receptors generally comprise the extracellular portions of membrane-bound receptors hence have the affinity to bind ligand (65). Unlike membrane bound receptors, binding of ligands to soluble form of receptors does not induce downstream signal cascade since they lack transmembrane and cytoplasmic domains. As the result, they antagonize the function of their respective membrane bound receptors (66). The soluble forms of receptors have

also important implications as disease markers, and believed to be potential therapeutic agents.

Soluble form of receptors can be formed either by alternative mRNA splicing that gives rise to a polypeptides lacking a transmembrane region, which is then secreted from the cell or by the proteolytic cleavage of membrane-bound receptor proteins from the cell surface into the extracellular fluid, or in to the supernatant in case of *in vitro* cultures (65, 66). The production of soluble receptors is interrelated to membrane bound receptors and also tightly regulated (67). Several soluble receptors are identified for different cytokines such as soluble form of Il-1R, IL-2R, IL-4R, IL-5R, IL-6R, INFγR and TNF-R, G-CSFR, GM-CSFR, LIFR, CSF-1R, etc. We focus on the soluble form of M-CSF and GM-CSFR.

1.3.10.1.Soluble form of GM-CSFR

As discussed above granulocyte-macrophage colony-stimulating factor (GM-CSF) and its receptor is essential in hematopoiesis and host defense. There is also soluble form of GM-CSF receptor (sGM-CSFR) identified recently in chorio-carcinoma cells. The membrane bound, and soluble receptors are identical except that the soluble receptors lack the transmembrane and cytoplasmic domain of the receptor. This secreted receptor retained its capacity to bind GM-CSF in solution and appears to play a regulatory role in the wide range of biological responses mediated by these cytokines by blocking it from binding to its membrane bound receptor (68).

1.3.10.2. Soluble form of colony stimulating factor-1R (CSF-1R)

A soluble form of CSF-1R was recently discovered in goldfish primary kidney macrophages and associated with decreasing proliferation and differentiation of the cells (19). It contains extracellular ligand binding domain but lacks transmembrane and cytoplasmic domain (68, 69). Recent studies (63,70) show that CSF-1R expressed in different groups of fish monocyte/macrophages cells. The fish CSF-1R has similar signal sequence, a transmembrane domain and a tyrosine kinase domain, all in conserved positions as in higher vertebrates. Though some pharmacological molecules that inhibit the action of CSF-1 and its receptor exist, no soluble form of CSF-1R has been discovered in higher animals yet. Therefore, studying of fish soluble form of CSF-1R can be a valuable model for defining the ancient strategies that were used by lower vertebrates for regulation of macrophage development and function. Alternatively, it may provide important insights about a strategy that has remained uncharacterized in higher vertebrates.

1.3.11. Significances of soluble receptors in therapeutics and clinical trials

As they are pivotal in the health and immune system, cytokines can also causes problems when they produced in excess or abnormally. Soluble cytokine receptors are essential in the regulation of cytokine related problems. They prevent cytokines from binding to their membrane bound receptors and exclude from generating a biological response (71,72). Hence, the soluble receptors might help to prevent or treat the diseases related to excess production of cytokines. On the other hand, the excess production of soluble receptors can also cause problems

when they interfere with the normal functions of cytokines. For instance, orthodontic tooth movement, arthritis, visceral leishmaniasis, Hodgkin's lymphoma, metastatic renal cell carcinoma, blastocyst implantation and breast cancer (73, 74) are some health problems that involve soluble receptors. Thus, the soluble receptors have dual effect: to induce or inhibit in cytokine related diseases (75).

1.3.12. Fish immune system

The increasing economic importance of fish has enhanced the interest in the study of defense mechanisms against fish infections and diseases. Understanding the fish immune system is of considerable interest in aquaculture and fisheries. It is vital in fish disease diagnosis, treatment and development of effective vaccines. Fish also serve as a model for studying the immune system in higher vertebrates and to as a bioindicator in environmental pollution studies (76). Different types of fish have different organs of immune system (77). For example, the jawless fish (lampreys and hagfishes) produce their immune cells in the anterior kidney (or pronephros) and some areas of the gut. Cartilaginous fish, on the other hand, have different specialized organs that house the immune cells; the epigonal organs that surround the gonads, the Leydig's organ within the walls of their esophagus, and a spiral valve in their intestine. They also possess especial thymus and a well-developed spleen and kidney. They have a more advanced immune system than the jawless fish. Like chondrostean fish, the major immune tissues of bony fish (teleostei) include the kidney (78), thymus, spleen and scattered immune areas within mucosal tissues such as in the skin, gills, gut and

gonads (79). Like the mammalian immune system, in teleost the myeloid cells are believed to reside in the spleen whereas lymphocytes are found in the thymus (80, 81).

Teleost fish are derived from one of the earliest divergent vertebrate lineages to have both innate and acquired immune systems (82). They are considered to be an ideal model to study the immune systems precisely because of their phylogenetic position that they represent a transition point on the phylogenetic spectrum between invertebrates that depend only on innate immunity and mammals that heavily depend on adaptive immunity. The adaptive immune systems of teleost have not been elaborated to the extent seen in mammals. Alternatively, they are devoid of many of the "classical" features of the adaptive system found in higher vertebrates. The teleost fish innate immune system on the other hand, relies on several factors, which appears in other vertebrates such as some chemokines and their receptors (eg. IL-8), cytokines (such as IL-1, interferons, TGF-beta, TNF-alpha), acute phase NK cell receptors, and toll like receptors (79,82). The major mechanisms of the teleost fish innate immune response are suggested to be similar to mammals, although it requires further studies. For example the type I interferon (IFN) system is an essential innate immune response that protects fish from some virus infections (83). Fish have also show some specific immune responses, though it is not complex as that of mammalian immune system for instance, the secondary response is relatively minor and there is no IgG in fish. Instead fish have IgM and recently IgD has been described but its function is not well known (84). However, different forms of

fish IgM and its observed flexibility of structure may compensate for a lack of IgG class diversity. The presence of multiple forms of key constitutive and inducible components, such as lysozyme, C3, alpha2-macroglobulin and C-reactive protein, which may enhance immune recognition show that the innate immune response of teleosts appears to be highly developed (85,86). Recent studies have demonstrated that the essential components of the mammalian immune system (macrophages, B and T lymphocytes, MHC, CDs, cytokines, interferon pathway) are present in fish. The discovery of fundamental immune mechanisms in fish uncovers the primordial vertebrate immune repertoire; while some unique adaptations also illustrate how a group undergoing adaptive radiation in response to specific constraints (87).

Generally, the fish immune system is not as well characterized as that of mammals, but recently it has received increased attention particularly as it relates to teleosts. Many components of the innate immune system are conserved between fish and mammals (88) and some recognition and signaling mechanisms for mammalian immune system appears to be present in fish. Innate immunity generally activated when the pathogen-associated molecular patterns (PAMPs) is recognized by pathogen recognizing receptors (PRRs), a number of which, mainly toll-like receptors (TLRs), have been characterized in fish (89).

Through the evolutionary process, the mononuclear phagocyte lineage, especially macrophages, and their function are well conserved. However, a few key differences have been identified. Macrophage-like cells can be found in almost all multicellular organisms. In recent years much has been learned about macrophage

development in teleosts from studies in animal models that include zebrafish, goldfish, catfish, turbot, seabream and rainbow trout (90). These studies show that the cytokine responsible for the growth, proliferation and survival of macrophage and their lineage, CSF-1 for fish is similar with that of the mammals both structurally and functionally (91).

1.3.12.1.Zebrafish immune system

For decades immunologists have relied heavily on mouse models for their experimental designs for the study of immunology. Recently, some fish species including the zebrafish (*Danio rerio*) have become powerful vertebrate models (90). This is highlighted in zebrafish, for example by recent advances in mutation screening techniques, the creation of genomic resources, and the development of efficient transgenesis procedures (92). Further, the zebrafish embryo provides a good developmental model because of its transparency (93).

In mammals, the hematopoietic system is established early during embryogenesis and is required for the continuous production of blood during fetal and adult life. Recently, zebrafish genetics have contributed towards the understanding of early blood and vessel development. In short, these studies point to the conservation of hematopoietic programs between mammals and teleost fish (94). The kidney is the main hematopoitic organ of zebrafish and they also have circulating leukocytes. Zebrafish have different forms of myeloid cells that include neutrophils, eosinophils, basophils and monocyte/macrophages (90). Like mammals, zebrafish hematopoiesis also shows two forms, the early hematopoiesis, which occurs during embryonic development and the definitive

hematopoisis, which occurs latter following the embryonic hematopoiesis (95).

acrophages play a vital role to the initiation and progression of host immune defenses in teleost fish. Many of these responses are highly conserved with those observed in higher vertebrates. For example, macrophages are well distributed throughout the teleost host and contribute to potent antimicrobial responses against invading pathogens through extracellular and intracellular killing responses. For instance, phagocytosis results in stimulation of the cell membrane and triggers the production of microbiocidal oxygen radicals or reactive oxygen species (ROS). Soluble stimulants, adherence of a pathogen to the macrophage membrane or other toxic substances that result in extracellular killing of pathogens may also cause release of ROS (101, 102). Like mammalian macrophages, zebrafish macrophages also express CSF-1R. Zebrafish macrophages can proliferate and differentiate in yolk sack without having functional CSF-1 receptor but do not move to other parts of the body like head tissues indicating that CSF-1 is important not only in proliferation but also differentiation and migration of the macrophages.

Zebrafish macrophage proliferation, differentiation, activation and migration are regulated by different cytokines and chemokines as in other vertebrate macrophages (96). Some of the cytokines that present in zebrafish includes PDGF family, interferon/IL-10 family, TNF family, IL-1 family and IL-17 family (97) and appears to exhibit the general mechanisms of recognition and cascade seen in mammals. There is a thought that the cellular components of fish blood differ from that of mammals because of their immune cells and

morphology. For example there are novel types of cells, which exist in teleosts but not in mammals. Furthermore, unlike mammalian B-cells, Fish B-cells appears to have phagocytic abilities. Zebrafish primitive macrophage lineage also appears to give rise to a number of neutrophilic granulocytes unlike that of mammalian primitive macrophage, which is considered to give rise to no other cell types (97). Recently more studies have been done on goldfish primary kidney macrophages and we will focus on that for detail review in this thesis.

1.3.12.2. Goldfish primary kidney macrophages

The goldfish primary kidney macrophages show three distinct subpopulations (98), which comprise of early progenitor cells, monocyte and macrophages. They express several differentiation markers, such as the hematopoietic stem cell antigen AC133, c-kit, granulin, CD63, macrosialin, c/EBPbeta, legumain, and the colony-stimulating factor receptor-1 (CSF-1R). The development from early progenitor cells to mature macrophages occur through either a classical pathways that follow early progenitor-monocyte –macrophage or alternative pathway that appears to bypass the monocyte stage and develop from the early progenitor to matured macrophages (99). In goldfish, there appears to be a strict control between self-renewing early progenitor cells, and those that were recruited into the maturation pathways (100). The sub-populations are different in their morphological, cytochemical and flow cytometric profiles, as well as in their antimicrobial functions. For instance, the early progenitors are small cells that contain acid phosphatase, but lack myeloperoxidase and nonspecific esterase. In contrast, both the mature macrophages and the monocyte are positive for acid

phosphatase, myeloperoxidase and nonspecific esterase. Each groups exhibited distinct functional responses in terms of production of nitric oxide and respiratory burst. The monocyte show early reactive oxygen intermediate production that decreases through time while the macrophages show some delay in production of reactive oxygen intermediates but continue to produce for extended periods (105). *In vitro* derived goldfish kidney macrophages pass through different growth phases. Lag phase is the early phase when the culture adapts to the new microenvironment. This phase is characterized by more cell death, which may be from mechanical damage during cell isolation and because of contamination with tissue fragment and other cells. Proliferative phase comes after the lag phase and is characterized by production of high-growth factors, high proliferation rate, and differentiation. As the result, the cell density increases and occupies all the available space. The nutrient also becomes exhausted, and the cell started to decease its proliferation by entering to senescence (106).

Cellular senescence is a stable form of cell-cycle arrest that may limit the proliferative potential of cells. There are different factors that derive the *in vitro* culture from proliferative phase to senescence phase like genetic and environmental factors or metabolic products. One of the genetic factors that induce senescence in cells is the telomere shortening after some repeated cell division (107, 108), which limit the number of replications. Senescence is generally characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased susceptibility to environmental effects and diseases. As the result, death is the ultimate consequence of senescence.

Recent studies in goldfish show that primary kidney macrophages produce endogenous growth inducing factors (102). Supernatants collected from proliferative phase cultures induce cell proliferation in freshly cultured *in vitro* derived goldfish kidney macrophages. On the other, hand supernatants collected from senescence phase goldfish primary kidney macrophage (PKM) cultures is a poor inducer of cell proliferation. Instead, it negatively impacts PKM proliferation (102). It has also been previously shown that goldfish PKM culture produce a novel soluble form of CSF-1R during the senescence phase and secrete into culture supernatants. As it is previously described, my objectives in this thesis were to analyze the effect of senescence phase supernatants from goldfish PKM cultures on further proliferation and survival of goldfish PKM cells.

1.2.14. Summary

Macrophages play key roles in immune system and homeostasis. They are essential for the detection of foreign antigens and malignancy. Their phagocytic, cytotoxic and intracellular killing capacities make them central contributors to the immune system. More specifically, they are essential in phagocytosis, antigen presentation, and clearing debris from the body, in embryonic development and wound healing. Macrophages can mediate phagocytosis through several mechanisms, including Fc receptor and complement-mediated phagocytosis. They also produce cytokines that affect their own function and that of other immune cells. In conclusion macrophages play a critical role in both innate and adaptive immune system, therefore, central to proper immune functioning and a target of multiple regulatory events. Macrophage proliferation, differentiation, survival and

key effector functions are mainly regulated by macrophage colony stimulating factor (CSF-1). This cytokine exists in both secreted and membrane bound form and exerts either autocrine or paracrine effects on the target cells. Upregulation of macrophage production, and activation for induction of the immune system, and/or down regulation of their production and activation, for inhibition of some inflammatory responses or malignancy can be possible through regulation of the cytokine or its receptors. There are some pharmacological products that negative regulate the function of this cytokine and its receptor. Recent studies in goldfish reveal that there is a soluble form of CSF-1R that expressed in goldfish PKM cells and has a potential to inhibit the function of CSF-1 and its receptor. Further, there are preliminary indications from zebrafish that point to the conservation of this soluble receptor among teleost fish. My thesis focuses on the effect of senescence phase supernatant on goldfish PKM proliferation and survival. This may help to get insights into the role of sCSF-1R, which exist in the senescence phase supernatant, in macrophage self-regulation. Soluble receptors can also use as indicators of diseases conditions. Hence, this study will have a significant contribution for the development of scientific knowledge.

1.2.15. References

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CHAPTER TWO: MATERIALS AND METHODS

2.1. Fish

Goldfish (*Carassius auratus*) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20°C in a flow-through water system on a simulated natural photoperiod and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments.

2.2. Cell lines

Sf9 insect cell line (isolated from ovarian tissue of the fall army worm, (*Spodoptera frugiperda*) was obtained from the lab of Dr. Mike Belosevic, Department of Biological Sciences, University of Alberta and used for the production of recombinant soluble form of colony stimulating factor -1 receptor (refer to sections 2.12 and 2.13 for details).

2.3. Culture media

The following media were used throughout this thesis work. MGFL-15, which has been described previously (1). Complete medium was prepared by adding 100 U /ml penicillin, 100 g/ ml streptomycin, 100 g /ml gentamicin, 10% newborn calf serum (Invitrogen Grand Island NY, USA) and 5% carp serum.

Serum free SF-900 II SFM (Invitrogen, Grand Island NY, USA) was used for the growth of the sf9 insect cell line. Goldfish primary kidney macrophage cell cultures were grown at 20^0C in non-vented culture flask in the absence of CO_2 and

insect cell lines were grown in 27^0C incubator without CO_2 using non-vented culture flasks.

2.4. Antibodies

Rabbit polyclonal antibody to 6x His tag, IgG1 (Thermo scientific) and goat anti rabbit IgG-HRP antibody (BIO-RAD) were used for Western blots (refer to section 2.15.3 for details).

2.5. Isolation of kidney leukocytes and establishment of PKM cultures

Goldfish primary kidney macrophages were isolated using procedures described previously (2). Briefly goldfish were taken from Aquatic Facility in the Department of Biological Sciences, University of Alberta and sacrificed. Kidney was taken and mashed on stainless steel screen using homogenizing solution (MGFL-15 medium, containing 100 U/ml penicillin, 100 μg /ml streptomycin antibiotics and 50 U/ml heparin). The solution was layered on 51% Percoll solution and centrifuged at 1450 rpm for 25 minutes. The early progenitor cells were isolated from the interface between the media and Percoll. The viability of cells was determined by trypan blue staining and counted by hematocytometer under inverted microscope (ECLIPSE TS100, Nikon, NY, USA). Finally, the cells were seeded either in 75 cm^2 or 25 cm^2 culture flasks at a density of 16-20 $x10^6$ and 7-10 $x10^6$, respectively (2).

2.6. Collection of cell conditioned media

Goldfish PKM growth phases were defined previously based on morphologic and flow cytometeric analysis (2). Cell conditioning medium (CCM)

was collected at either proliferative (approx. day 5-7) or senescence (approx. day 9-13) phases supernatant. The average days were taken to accommodate for variability in fish cell development. A combination of culture morphology and flow cytometric characteristics was used to define the times for individual supernatant collections. The CCM was collected for proliferative phase when the PKM cultures appeared morphologically round, less debris, less adherent and more non adherent cell populations, low or no vacuolization and have good proportion of the three macrophage subpopulation. On the other hand, senescence phase CCM was collected when the cultures had more adherent cells with expanded cytoplasm, more debris or vacuolization, and relatively less non-adherent cells and more clumps (2-5).

2.7. Treatment of proliferative phase primary kidney macrophages with different phases of cell conditioned media (CCM)

Goldfish primary kidney macrophage progenitor cells were cultured in complete MGFL-15 media (10% FBS and 5% carp fish serum) for five to seven days with proliferative phase CCM collected from previous PKM cultures. The non-adherent cells from proliferative phase culture were centrifuged at 1100 rpm for 10 minutes and supernatant was removed (unless specified, we used non-adherent cells in all our experiments). The pellet was re-suspended in new media and divided in to three different flasks to treat with senescence (50% senescence phase CCM and 50% new media), self (100% self CCM, no new CCM and no new media) and proliferative (50% proliferative phase CCM and 50% fresh medium). 50% CCM treatment was used based on the result from the treatment

for optimization using 25%, 50% and 75% CCM and 75%, 50% and 25% new media, respectively.

2.8. Analysis of goldfish primary kidney macrophage culture morphology

Culture morphology analysis was based on observation using inverted microscope (ECLIPSE TS100, Nikon, NY, USA) using the whole culture flask for adherence and non-adherence population, presence of debris, formation of clumps, formation of island, media color, single cell morphology and density of the cell in the flask.

2.9. Analysis of cell death in goldfish primary kidney macrophages after treating with different phases of cell conditioned media

Non-adherent cells from proliferative phase PKM cultures were centrifuged and re-suspended in new medium and treated with different phases of CCM. On day 4 post treatment, the cells were collected by centrifugation at 1100 for 10 minutes, washed twice with 1×$PBS^{-/-}$ (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO and 2 mM KH_2PO_4) and re-suspended in 1× Annexin V buffering solution (5x Annexin V buffer solution; 50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4,Invtrogen, Grand Island, NY, USA) was diluted to 1x before use). The cells were diluted to 1µ10^6/ ml and then sample was taken and stained with AnnexinV Alexa Flour 488 and 7AAD (10 µg/ml) for 15 minutes and analyzed by FACS (FL-1 for Annexin V Alexa Flour FL-3 for 7AAD).

2.10. Analysis of cell death through out the growth phases of goldfish PKM cultures

The same procedures were followed for the analysis of apoptosis and necrosis through out the growth phases of the cell culture as in section 2.9. This was designed to have baseline information about the trend and extent of cell death in regular culture condition in each phase.

2.11. Comparison of chemical sensitivity between proliferative and senescence phase PKM cells

Proliferative and senescence phases of goldfish PKM cells were centrifuged, supernatant discarded and the pellets were re-suspended to the concentration of 1×10^6/ml. Then the cells were treated with H_2O_2 in the final concentration of 0.0882 mM (Research grade 30% w/v H_2O_2, 8.82 mM solution EMD, Gibbstown, USA) at room temperature for 30, 60, 120 and 180 minutes. Optimization of the treatment time was done by incubating for 30 minutes, 1, 2, 3, 4, 6 and 24 hours before choosing 180 minutes as treatment time limit. After treatment time, cells were centrifuged, and the pellet was re-suspended in FACS buffer solution (1xPBS$^{-/-}$ and 5% FBS). Finally, the cells were stained with 7AAD for 10 minutes and analyzed by FACS using FL-3H and FSC-H. The types of cell death induced by H_2O_2 were also analyzed by using Annexin V Alexa Flour 488, for apoptosis and 7AAD, for necrosis. Goldfish PKM cells were treated with the same concentration of H_2O_2, and the types of cell death are analyzed.

2.12. Amplification and purification of expression plasmid

The recombinant soluble form of the CSF-1R (rsCSF-1R) was expressed using an insect-based protein expression system. We obtained previously cloned plasmid (6) from Dr. Mike Belosevic's lab (Department of Biological Sciences, University of Alberta) and amplified using *E. coli*. The cloned plasmid was transformed in to chemically competent TOP10 *E. coli* (Invitrogen, Grand Island, NY, USA) according to manufacturer's specifications. Once the positive clones were isolated, restriction digest followed by gel electrophoresis verified the presence of insect and vector DNA. Plasmids were sequenced in order to confirm that the inserts were ligated in to the expression vector in the proper orientation and open reading frame. Sequence data was analyzed using Gene tool (Biotools Jupiter, FL, USA) and Gene Construction Kit 2 (SciQuest) software package (6). After the proper transformation, a single positive colony of bacteria was transferred and grown in the LB media containing Ampicillin at 37^0C for 7 hrs on shaker at 225 rpm. The QIAprep kit was used to purify plasmid from the TOP10 *E. coli* bacteria according to the manufacturer's specification. The principle of the QIAprep method is to use alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica-gel membrane in the presence of high salt. Then the DNA is washed followed by eluting from the membrane.

2.13. Cell transfection, selection of stable cell lines and production of recombinant goldfish soluble form of the CSF-1R

The sf9 insect cells were grown in serum free SF-900 II SFM media (Invitrogen) at 27^0C. When the cells form monolayer and the viability is about 95%, cells were transfected using Cellfectin reagent (Invitogen) according to the manufacturer's specification. Generally $1x10^6$ cells in 6 well plates were transfected with 1 μg sCSF-1R expression plasmid. After 3-4 days, stable transfect were selected with blasticiden (Invitrogen Grand Island NY, USA) by starting with 50 μg/ml for two round cultures and 30 μg/ml for another two round cultures. Finally the cells were grown in media containing 10 μg /ml blasticidin for 5-7 days and then the supernatant was collected.

2.14. Purification of recombinant goldfish soluble CSF-1R

The recombinant protein was purified from the cell culture supernatant or cell lysate by 6x His-tagged protein purification kits using Ni-NTA metallic chelating according to manufacturers specification. The MagneHis™ Protein Purification System uses paramagnetic precharged nickel particles (MagneHis™ Ni-Particles) to isolate polyhistidine-tagged protein from cell lysate or supernatant. Briefly, cells were lysed using Fast break cell lysis reagent (Promega Fitchburg, WI, USA) according to manufacturers protocol. Then the cell lysate or supernatant collected from the cell a culture was concentrated by using polyethylene glycol and dialyzed overnight against $1xPBS^{-/-}$. The Ni-NTA particle was added to the concentrated sample and the paramagnetic particles were precipitated by magnetic stand, and then carefully removed from the solution. The

Ni-NTA partcles were washed three times with washing buffer (40 mM Immidazole and 100 mM HEPES). Finally the bound protein was eluted from paramagnetic particles by elusion buffer (500 mM Imidazole and 100 mM HEPES). The solution was then dialyzed overnight against 1xPBS$^{-/-}$ to remove the elusion buffer from the protein.

2.15. Immunodetection of rsCSF-1R expressed in insect cell lines

2.15.1 BCA protein assay

Bicinchoninic acid protein assay is the colorimetric method that helps to determine and quantify the total concentration of protein in the solution. Protein concentration was analyzed by using BCA protein assay kits, which includes BCA Reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, BCA Reagent B, containing 4% cupric sulfate and Albumin Standard Ampules, containing bovine serum albumin (BSA) (Thermo scientific) according to manufacturer's specification.

2.15.2. Coommassie blue and silver stains

Coomassie blue staining solution was prepared by mixing 0.2% Coommasie blue, 50 % methanol and 10% glacial acetic acid. The destaine solution was prepared by mixing methanol and acetic acid .The gel was socked to the staining solution for 60 minutes with agitation. Excess dye was washed by destaine solution (the same solution but with out Coomasie blue dye) for an hour with continuous agitation.

The silver stain was done according to manufacture's specification (BIO-

RAD, Alfred Nobel Dr., Hercules CA). Briefly, the gel was fixed by fixative solution (Methanol Reagent Grade 50% Acetic Acid 10%, Fixative Enhancer Concentrate 10% and Deionized Distilled Water 35% v/v) for 20 minutes. The gel was rinsed twice in distilled water for 10 minutes each. Then stain with stain solution (prepared by mixing Milli-Q water, silver complex solution, reduction moderator solution, image development reagent according to the manufacturer's specification) for 15-20 minutes. Finally, the gel was put in 5% acetic acid for 15 minutes to stop the staining reaction.

2.15.3. Western blot

SDS-PAGE buffers; 10% separating gel buffer (30% Polyacrylamide, 1.5 M TRIS PH 8.8, 10% Ammonium persulphate and TEMED), 4% stacking gel buffer (30% Polyacrylamide, 0.5 M Tris PH 6.8,TEMED and 10% Ammonium persulphate), Running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), and Blocking buffer (5% dry milk in TBST) were made as necessary for running SDS PAGE. The sample proteins were loaded to the gel and the gel was run at 100 V for 20 minutes and at 185 V for 40 minutes. The protein band was then transferred to nitrocellulose membrane from the gel, by electrophoresis. The membrane was stained with Ponceau staining solution to ensure that the protein was transferred. The nitrocellulose membrane was incubated with blocking buffer (5% dry milk in 1x PBS). Then the membrane was incubated with primary antibody (Rabbit anti-His polyclonal antibody diluted 1:500 in 2.5% blocking buffer) for 1 hour. After washing the membrane three times with 1xPBS$^{-/-}$ it was incubated with secondary

antibody (Goat Anti-rabbit IgG-HRP Antibody diluted 1:1000, BIO-RAD laboratories, Alfred Nobel Dr., Hercules, CA) for 1 hr. Then the membrane was washed three times with 1x $PBS^{-/-}$, each time for 10 minutes. Finally the protein was detected by using ECL Western blotting system. The two ECL detection reagents (GE Healthcare, Buckinghamshire, UK) were mixed in equal volume and the membrane was soaked in the detection reagent for one minute. This elicits a peroxidase-catalyzed oxidation of luminol and subsequently enhanced chemiluminescence, where the HRP labeled protein is bound to the antigen on the membrane. The resulting light was detected on Hyperfilm ECL Western after exposing the film to the membrane for one minute in dark room.

2.15.4. Mass spectrometry

The identity of protein was confirmed by mass spectrometry in the Department of Chemistry, University of Alberta. Proteins in the gel were reduced with 5 mM DL-Dithiothreitol and carbamidomethyllated with 10 mM iodoacetamide followed by tryptic digestion overnight with 0.06 µg/µL modified bovine trypsin (Promega) at 30^0C. The resultant peptide digests were extracted using 20 µL of 50% acetonitrile, 0.2% formic acid in water for 2-3 times. The pooled extract was then dried to about a third of the volume in a speed vac. Then the resultant peptides were subjected to LC-MS/MS analysis on a UPLC (Waters, Milford, MA coupled with q-TOF premier mass spectrometer (Water, Milford, A). Obtained MS/MS data were analyzed through proteomic software called Mascot (Version 2.2, Matrix Science).

2.16. Analysis of the inhibition effect of senescence phase supernatant and rsCSF-1R on the PKM cell proliferation

The BrdU cell proliferation assay was done for both PKM treated with different phases CCM and rsCSF-1R. Proliferative phase PKM culture was treated with different phases of CCM and rsCSF-1R (10ng/ml) for 4 days. Cell proliferation assay was done using Cell proliferation (colorimetric) BrdU assay kit (ROCHE) for all PKM cultures according to manufacturer's specification and the absorbance was taken at 450nm wavelength using VERSAmax micro plate reader.

2.17. Statistical analysis

The statistical analysis of the data was evaluated by unpaired two-tailed t-test (Graphpad quickCalcs Software). P values of <0.05 was considered significant.

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CHAPTER THREE*: IN VITRO* GENERATION OF GOLDFISH KIDNEY MACROPHAGES

3.1. Introduction

As discussed in chapter 1, the goldfish PKM cell cultures show three different subpopulations, which develop spontaneously in response to endogenous growth factors (1). These sub populations correspond to early progenitor cells, monocyte and mature macrophages. The early progenitor cells can develop to mature macrophages either through classical pathway, which follow the early progenitor-monocyte line and develop to mature macrophages or the alternative pathway, which appears to bypass the monocyte stage and develop from the early progenitor directly to mature macrophages (1, 2). Hence the early progenitor cells can either self renew or enter the maturation pathways (3) and this process is strictly regulated

The three subpopulations` exhibit some distinguishing markers (4). They have distinct morphological, flow cytometrical, and cytochemical profiles. Based on flow cytometric characterization using FSC-H for size and SSC-H for granularity, the early progenitor cells are smaller in size and show low granularity while mature macrophages are larger in size and have more internal complexity. They also have functional differences. For example, the early progenitor cells are positive for acid phosphatase but negative for myeloperoxidase and nonspecific esterase. On the other hand, both the mature macrophages and monocytes are positive for acid phosphatase, myeloperoxidase and nonspecific esterase. Furthermore, the monocytes and macrophages also have distinct functional

responses in terms of production of nitric oxide and respiratory burst. The monocytes show early production of reactive oxygen intermediates, which is not maintained for long periods of time while the macrophages show some delay in the beginning the in production of reactive oxygen intermediates but continue to produce for extended periods (5).The proportion of the subpopulations varies at different growth phases of the culture. Therefore, understanding the growth phases of the culture and the composition of each phase is important for further analysis of their production and function.

In vitro derived goldfish kidney macrophages pass through different growth phases during their growth period; lag phase, proliferative phase and senescence phase. The lag phase starts when the cells first inoculated, and it is the phase of adaptation to the new microenvironment. Once the debris and other tissue fragments are cleared, and the macrophage subpopulations acclimatized to the environment and start growing, the cells enter the proliferative phase. This phase is characterized by the rapid proliferation and growth of the cells, maximum rate in the production of growth factors, and cell differentiation. Following the proliferative phase is the senescence phase where production of growth factors decreases, and cell proliferation rate declines (6,7). Instead, the production of the inhibitory factors increases. Therefore, these growth phases are different in the proportion of each subpopulations as well as the composition of their supernatant. This appears to be particularly the case for growth-promoting and inhibitory factors, which are our focus in this study.

Different factors such as genetic and environmental factors can shift the *in*

vitro cultures from proliferative phase to senescence phase. Cellular senescence induced by genetic factors is due to telomere shortening after some repeated cell division (8), which limits the number of replications (9). In chemically defined medium, the nutrient depletion that decreases the carbon and nitrogen source also leads the culture to senescence (10). As the cells grow, the accumulation of metabolic products, apoptotic bodies and cell density also increase in the cultures. The cell response to these different environmental stresses might be either apoptosis or entering to senescence (11,12). Cellular senescence is one of the mechanisms that arrest cell cycle, and limit the proliferative capacity of cells. This is particularly important to limit the premalignant cells *in vivo*. Senescence is characterized by increasing homeostatic imbalance, declining the ability to respond to stress, and increased susceptibility to environmental effects and diseases. As the result, death is the ultimate outcome of senescence (7, 12,13,14). In this thesis I am interested to analyze whether the goldfish PKM cells secrete endogenous growth inhibitory factors that negatively impact the cell survival and proliferation.

3.2. Objectives

Cellular factors secreted into PKM supernatants in lag, proliferative and senescence phases appear to vary significantly, and are predicted to differ with regards to immune-modulatory factor composition. The main objectives of the study in this chapter were to establish, and characterize the different phases of goldfish PKM cell cultures. The goal was to minimize cross-CCM functional differences in order to allow for preliminary characterization of CCM activity. These supernatants subsequently serve as important tools for characterization of

goldfish PKM cells endogenous regulatory mechanisms, which limit their proliferation and survival.

3.3. Results

3.3.1. Morphologic and flow cytometric characterization of goldfish primary kidney macrophage (PKM) cell cultures

The flow cytometric analysis with forward scatter (FSC-H) for size and side scatter (SSC-H) for granularity shows that the PKM cultures have three subpopulations (1). These three subpopulations exhibit phenotypic differences (Figure 1). Flow cytometric analyses are supported by ImageStream analyses, which further illustrate the morphological differences that exist among these three unique PKM subpopulations (15). The results show that early progenitor cells are smaller in size and have lower granularity than both monocytes and macrophages (Figure 1B). On the other hand, macrophages are larger in size than both monocyte and early progenitor cells. As expected, I was able to recapitulate the three growth phases identified in PKM *in vitro* cultures (lag phase, proliferative phase and senescence phase) based on flow cytometric analysis and microscopy-based analysis of PKM *in vitro* cultures.

3.3.2. Effect of senescence phases supernatant on goldfish primary kidney macrophage culture morphology

Goldfish PKM cultured using regular culture protocol and allowed to grow until proliferative phase as in section 3.2.1. Non-adherent cells from proliferative phase were subdivided for treatment with self-CCM, proliferative phase CCM and senescence phase CCM for four days. On day four, the cultures were analyzed using microscope (ECLIPSE TS 100 Nikon, NY, and USA) at 200x magnification. The microscopic analysis reveals that cultures treated with

senescence phase supernatant show the features of the senescence phase based on the criteria we used to track culture morphology. These include low cell density, more adherent populations, more clumps and islands, and extended cytoplasm, and most cells have an irregular shape. We also analyzed to see if the phenotypical changes are due to some change in the media composition as the result of prolonged incubation in warmer temperature (20^0C), which subsequently affects the cell cultures. The main concern in this experiment was the decomposition of glutamine, which is very important for the cell to synthesis protein and nucleic acids (16). To test this, we first pre-incubated the media without cells for 14 days at 20^0C, which corresponds to the period and temperature of senescence phase cultures. Then we incubated the cells with this pre-incubated media to determine if it has similar effects on the cell culture as the senescence phase supernatant. The results showed that cells in the pre-incubated media grow well (Figure 3.3), and there were no morphological changes equivalent to those observed in the cultures treated with senescence phase supernatant.

3.3.3. 7AAD for assessment of viability in goldfish primary kidney macrophages

Proliferative phase PKM cultures were stained with 7AAD for different times as described in the materials and methods (Chapter II). Flow cytometric analysis based on FL-3H for 7AAD and FSC-H (size) showed that 7AAD stain the cells within the first five to ten minutes. Furthermore, analysis from staining the cells with 7AAD after inducing cell death by H_2O_2 showed that 7AAD

discriminate between dead and live cells in goldfish PKM (Figure 3

3.4. Discussion

The goals for experiments described in this chapter were to establish PKM cell cultures and characterize the growth phases during the growth periods of the cultures. In addition, I also optimized protocol to use 7 AAD for viability test in goldfish PKM cells for further experiments. I established the goldfish kidney leukocyte cultures, which show different growth phases as it was described previously (1- 5). These growth phases of goldfish PKM cultures indeed showed different features based on their cell composition, cell size, density of the cell (Appendix A) and secretion of endogenous growth inducing factors as previously described (3). Flow cytometric analysis based on forward scatter (FSC-H) and side scatter (SSC-H) show that lag phase of culture predominantly contains early progenitor cells while the proliferative phase cultures, which ranges from day 6-9 of *in vitro* cultivation, contain all the three-sub populations in relatively considerable number. In senescence phase, both FSC-H and SSC-H parameters change and reflect an increase in size and cellular complexity. Interestingly, ImageStream analysis also supports the flow cytometric profile, and shows that macrophages are larger in size, while the early progenitor cells are the smallest of the three (4). Microscopic analyses of culture morphology also show that the different phases of goldfish PKM cultures have distinct phenotypes. The lag phase cultures have some floating debris, low density and smaller cells. The proliferative phase cultures have more non adherent cells, low debris, small

adherent cells and low vacuolization whereas the senescence phase cultures have more adherent cells, clumps, islands, amorphous cell morphology and extended cytoplasm and larger cells. These different characterizations of the goldfish macrophage growth are similar with previous studies in different species (1-5). Characterization and establishment of protocols for the consistent generation of PKM cultures at these distinct phases of macrophage *in vitro* development allowed me to begin to assess potential differences between supernatants derived from each of these phases. These cell-conditioned media (CCM) samples were collected, and stored at 4^0C in preparation for further experiments. If properly stored, the CCM can be used at least for one year without losing its activities based on our experience and filtration is recommended before storage.

The goldfish PKM cultures treated with senescence phase CCM show the features of senescence cultures. These phenotypic change from the treatment of senescence phase supernatant is not due to change in the media as the result of prolonged incubation in warm temperature (20^0C) because PKM treated with media pre-incubated for 14 days at the same temperature as the culture but without cells, grew like cells cultured in regular media. This show that pre-incubation of the media alone does not affect the growth of the culture. This experiment was done to see whether the pre-incubation affect the media composition such as L-glutamine. L-glutamine is amino acid, which is important for the growth of cells in the culture but it is unstable and can be decomposed in the media (16,18,19,20). My results show that the pre-incubation did not affect the PKM growth suggesting that the pre-incubation alone does not affect the PKM

growth but it is not clear whether the pre-incubation does not decompose the L-glutamine or the goldfish PKM may not be sensitive to the decomposition of glutamine in the media. It needs more work to see the composition of media after pre-incubation besides functional analysis. The result also strongly suggests that the senescence phase supernatant contains growth inhibitory factors that affect the growth of the culture and alter the culture phenotype.

7-aminoactinomycine D is fluorescence chemical with strong affinity for DNA. 7AAD does not enter cell with intact cell membrane. When the cell membrane integrity compromised, it enters the cell and stain DNA. As a result 7AAD is commonly used as a viability test in mammalian cells to discriminate between live and dead cells. My results show that it also discriminates between live and dead cells in goldfish PKM cells. This is the first report in goldfish PKM cells to my knowledge and it can be used as viability test in fish cells. During early apoptosis, cells` membrane integrity is maintained excluding 7AAD from the cells, but during necrosis, the cell loss its membrane integrity, which allows the 7AAD to enter to the cell and stain the nucleus (21). On the other hand, phosphotydilsyrine (PS) flips from inner membrane to outer membrane during apoptosis. As the result, Annexine V will get access to bind to the PS. I used Annexin V Alexa Flour 488 and 7AAD for further analysis of apoptosis and necrosis respectively to differentiate the types of cell death induced in the goldfish PKM. This is important to understand how the PKM cells regulate themselves and how they respond to senescence phase supernatant to assess whether the senescence phase supernatant induce cell death through apoptosis, which is a

regulated process that can be cleared by phagocytes or necrosis, which is pathological and cause inflammation.

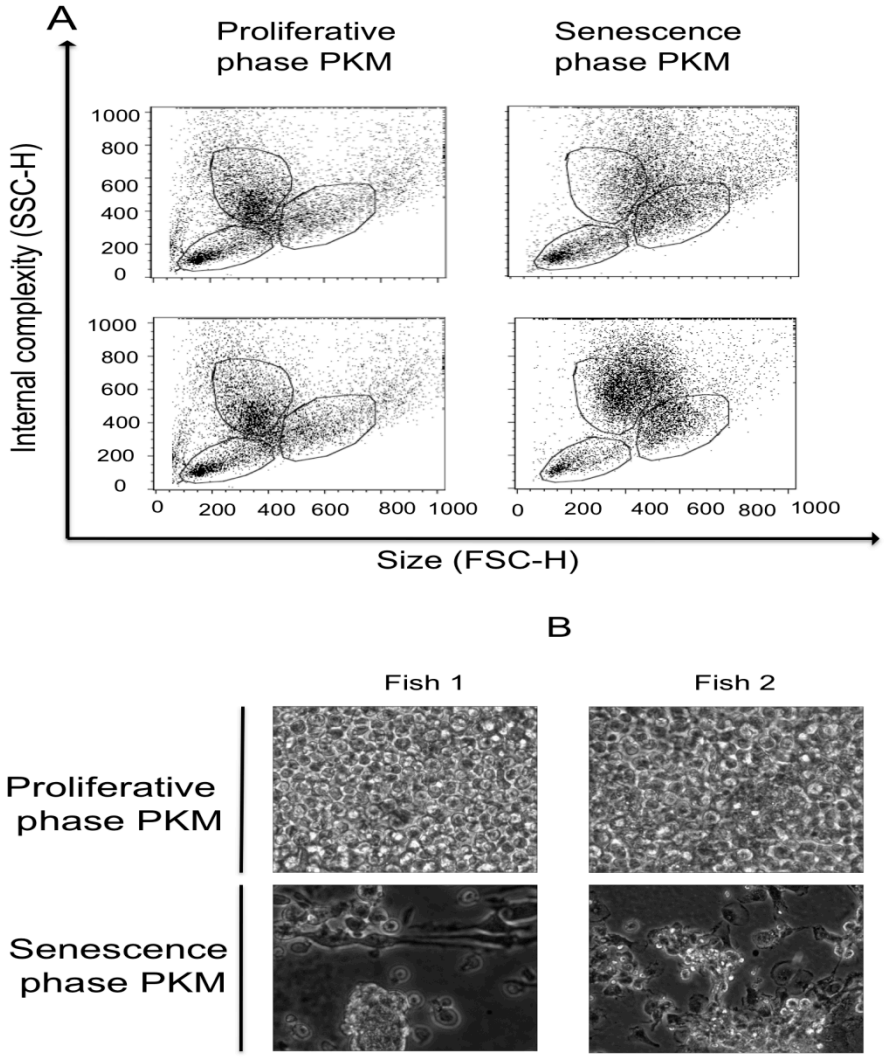

Figure 3.1. Flow cytometric and microscopic analysis of goldfish PKM cultures. Goldfish PKM progenitor cells were cultured with proliferative phase CCM at a density of 16-20x10^6 in 75 cm^2 or 8-10x10^6 cells in 25-cm2 non-vented culture flask and allowed to grow in 20^0C incubator. The cultures were analyzed by flow cytometry using FSC-H for size and SSC-H for granularity (A), and cells from proliferative and senescence phase PKM culture were analyzed by inverted microscope at 200x magnification (Nikon TS 100 microscope) to illustrate the culture morphology for the two phases (B). Figures for two representative fish are shown for proliferative and senescence phases (n=6).

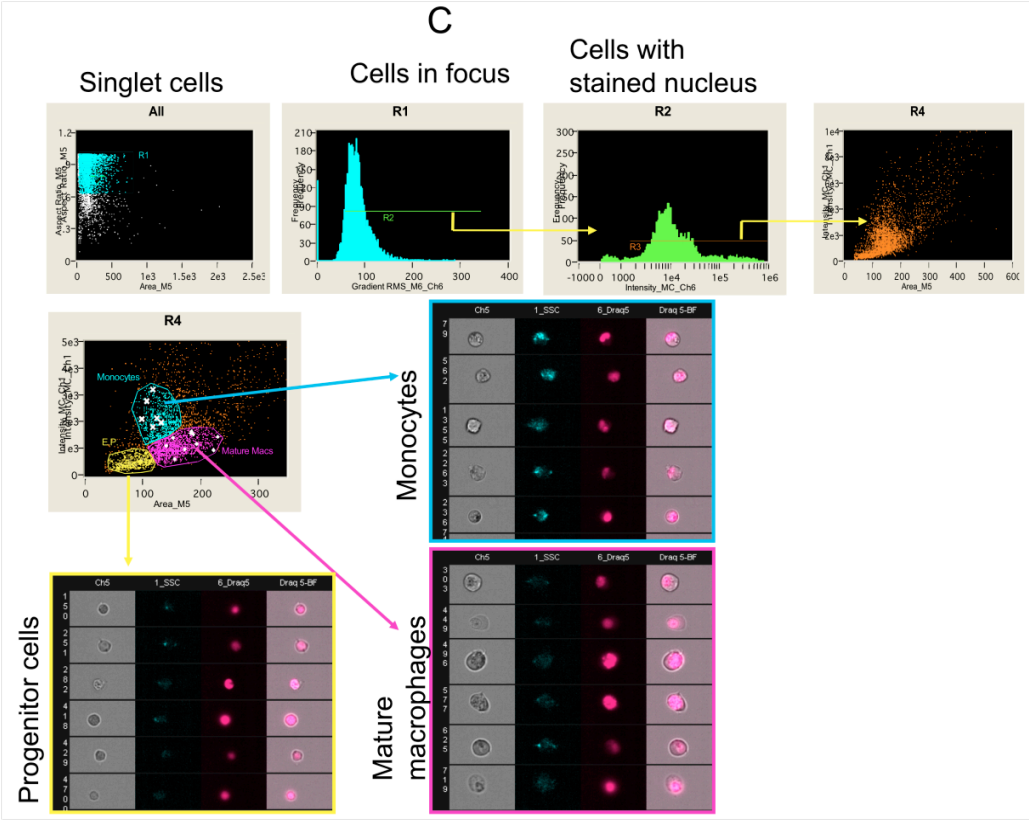

Figure3.2. ImageStream analyses of goldfish PKM cells. Cells were analyzed by ImageStream to see the primary kidney macrophage subpopulations and their morphological difference. R1 is singlet to remove clumps and debris, R2; cells in focus, R3 cells with stained nucleus and R4 are the macrophage subpopulations.

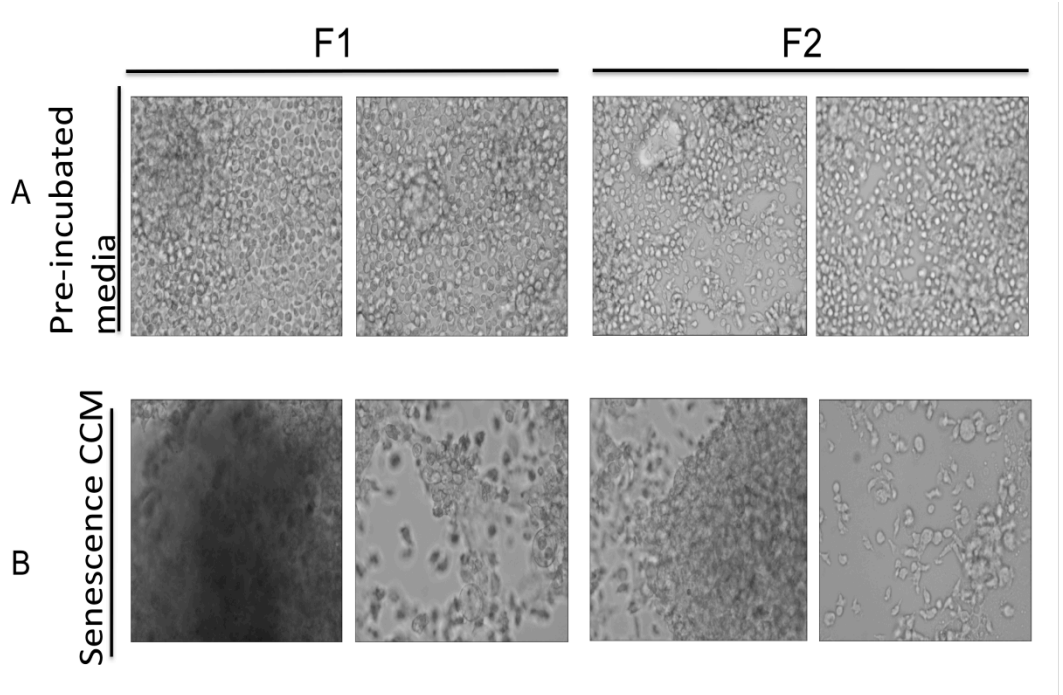

Figure 3.3. Microscopic analysis of PKM cells after treating with senescence phase CCM. The top figures (pre-incubated media) show the proliferative phase PKM cells treated with pre-incubated media (the media was pre-incubated at 20^0C for 13 days without cells) and analyzed by inverted microscope at 200X magnification (Nikon TS100 Microscope). The bottom figures (senescence CCM) show the proliferative phase PKM cells treated with senescence phase CCM for four days and analyzed by the same microscope. Duplicate pictures were taken for each phases of PKM from the same culture. Pre-incubation of the media was required to see whether the effect of the senescence phase CCM on PKM cultures is due to some change in the media condition as the result of prolonged incubation in high temperature or the endogenous factors that released from the cell to the media. Figures for two fish are shown (n=4).

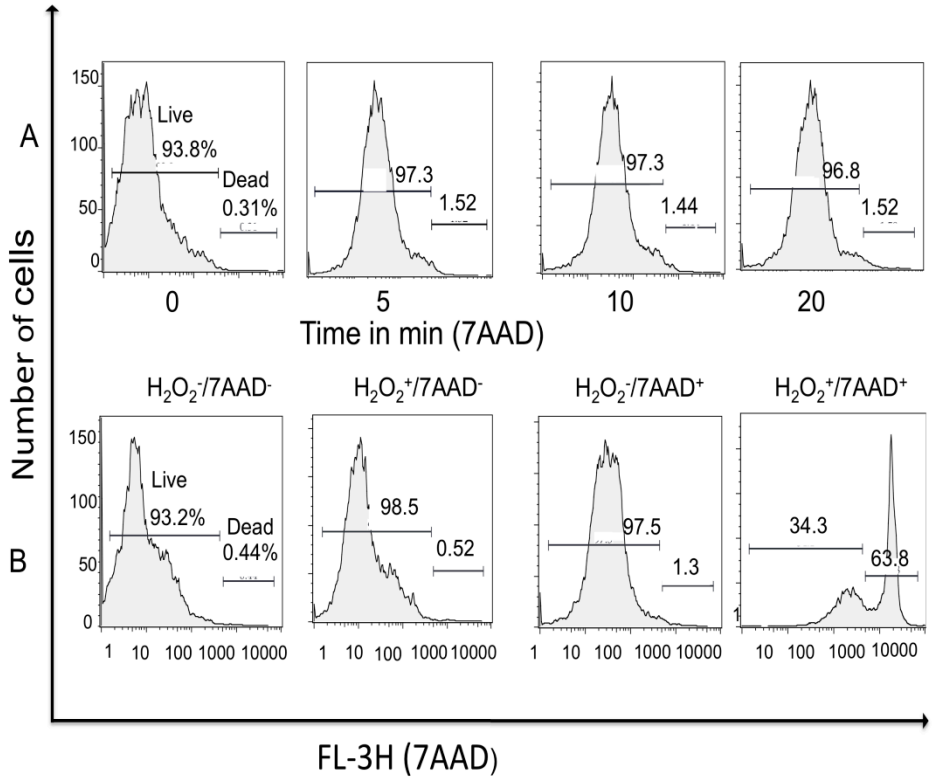

Figure 3.4.Application of 7AAD for viability test in goldfish PKM cultures. A) Samples (400 µl) of untreated goldfish PKM cells ($1x10^6$) were stained with 1 µl 7AAD (10 µg/ml) for different time courses. B) PKM cells were treated with H_2O_2 (final concentration of 0.0882 µM) for three hours to induce cell death. Treated cells were stained with 7AAD for 10 minutes and analyzed by flow cytometery. Representative figures are shown (n=4).

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CHAPTER FOUR: IMPACT OF SENESCENCE PHASE SUPERNATANT ON GOLDFISH PKM DEVELOPMENT

4.1. Introduction

In chapter three factors that derive the *in vitro* cultures from proliferative to senescence phase were discussed. I also established the proliferative and senescence phase PKM cultures and collected respective supernatants for further analysis. In this chapter I focus on the effect of senescence phase supernatant on goldfish PKM cell proliferation and survival, to provide a broad overview of the potential endogenous growth inhibitory factors that are produced by the PKM cells to down-regulate their own proliferation and survival.

Every cell has a life span and many cells can proliferate and bring descendants of the same cell types while some cells are able to differentiate into different cells. This proliferation and differentiation should have a limit and counterbalanced by cell death (1-3). Furthermore, cell death is part of normal development and maturation cycle and is the component of many response patterns of living tissues to xenobiotic agents (i.e. microorganisms and chemicals), to endogenous modulations (such as inflammation and unstable blood supply) and depletion of nutrient in cultured cells. Cell death is also a key variable in cancer development, prevention and treatment (4). Therefore, we are analyzing if the PKM produce endogenous soluble negative regulators to down regulate their proliferation and survival.

Broadly cells die in two ways: apoptosis or programmed cell death and necrosis. Apoptosis or programmed cell death (PCD) is essential in both development and homeostasis. It removes unnecessary, transformed, infected, or damaged cells by activation of an intrinsic, self-destructing program. It occurs under normal physiological conditions (5). It is common during normal cell turnover and tissue homeostasis, induction and maintenance of immune tolerance, embryogenesis, development of the nervous system and endocrine-dependent tissue atrophy (6). It results in shrinkage of cells, loss of membrane symmetry, condensation of nuclear chromatin, and formation of apoptotic bodies'. Since the apoptotic bodies remain membrane bound, phagocytosis of apoptotic bodies by phagocytes does not induce inflammatory reactions. Necrosis, on the other hand, is a pathological cell death, which is caused by extrinsic signals. It involves cellular swelling, denaturation and coagulation of cytoplasmic protein or rupture, and breakdown of cell organelles including nuclear DNA (7, 8). I analyzed the types of cell death in PKM treated with different phases of CCM to understand how the goldfish PKM cells manage their own cellular death. The analysis of cell death in this thesis was based on the change in membrane symmetry, which results in the externalization of phosphatidylserine, and the loss of membrane integrity that exposes the DNA and make accessible for the staining dye. Annexin V Alexa Flour 488 binds to PS and 7AAD binds to DNA, hence used to differentiate early apoptosis and necrosis.

4.2. Objectives

In chapter three, we characterized the goldfish PKM cultures growth phases and established stable protocols for collection of supernatants from different phases of PKM cultures. In this chapter I focused on the effect of senescence phase supernatant on PKM proliferation and survival by: 1) analyzing the effect of senescence phase supernatant on proliferative phase PKM cultures. In short, I determined whether the supernatant induce early shift of proliferative phase PKM to senescence phase, and whether cell death was induced by the senescence phase treatment. 2) Compared the chemical tolerance of senescence and proliferative phase goldfish PKM to see if senescence phase supernatant affects the ability of the cells to withstand environmental stress. This is important to understand how senescence affects the inflammatory response of macrophages as they produce reactive oxygen species during phagocytosis. 3) Investigated the effect of senescence phase supernatant on cell proliferation, to determine whether it inhibits the cell proliferation. The purpose of this experiment was to assess whether the senescence phase supernatants contain some growth inhibitory factors that inhibit cell proliferation. We also analyzed the effect of rsCSF-1R on goldfish PKM proliferation to compare the result with the effect of senescence phase supernatant, which enable us to investigate the potential contribution of native soluble form of CSF-1R, which has been previously found to be primarily derived from PKM senescence phase supernatants *in vitro* (9).

4.3. Results

4.3.1. Senescence phase supernatants induce early senescence in goldfish primary kidney macrophages

The cell-conditioned media (CCM) from senescence phase of goldfish PKM cultures have limited macrophage growth inducing factor activity (10). I hypothesized that this CCM also have some growth inhibitory factor(s) that negatively regulate the proliferation and survival of PKM cultures, and promote early shift of proliferative phase PKM to senescence phase. Among others, this may correspond to biological activity of the novel soluble form of the CSF-1R (sCSF-1R). Non-adherent cells from proliferative phase PKM cells were subdivided and treated with different phases of CCM for four days as explained in materials and methods (Chapter II). The growth of the cultures was monitored by microscopy based on morphological parameters and samples were taken on day 2 and 4 post treatment for flow cytometric analysis. The results from flow cytometric analysis based on forward scatter (FSC-H) and side scatter (SSC-H) characteristics showed that senescence phase CCM induced an early shift from proliferative phase to senescence phase relative to proliferative phase CCM and self-CCM treated cells, which remained in the proliferative phase during the course of treatment (Figure 4.1). Furthermore, the development of a prominent mature macrophage population within the corresponding flow cytometric gate suggested that senescence phase supernatants may also contain a higher content of macrophage maturation factors when compared to the culture treated with proliferative phase CCM and self-CCM (Figure 4.1).

4.3.2. Senescence phase supernatant induce early cell death in goldfish primary kidney leukocytes

The PKM supernatants represent a complex mix of molecules. My results re-enforce the concept that the senescence phase appears to be dominated by an increased production of growth inhibitory factors. However, there does not appear to be a clear kinetic boundary in regards to the production of macrophage growth and inhibitory factors. As such, senescence phase supernatants appear to contain a "net" inhibitory effect towards macrophage proliferation, but also appears to have a greater relative amount of macrophage maturation factors relative to proliferative phase supernatants. The results show that senescence phase CCM induces early cell death in the proliferative phase PKM cultures. Non-adherent cells from proliferative phase PKM cells were treated as in section 4.3.1. Flow cytometric analysis of cell death with Annexin V Alexa Flour 488 for apoptosis and 7AAD for necrosis showed that the senescence phase CCM induce earlier cell death compared to proliferative phase CCM, and this is mainly characterized by apoptosis. Figure 4.2 only shows the effect of senescence phase on PKM cell viability (the cell death show the total cell death both through apoptosis and necrosis).

4.3.3. PKM senescence phase CCM promote apoptotic cell death

Goldfish PKM progenitor cells were subdivided and cultured based on regular culture protocols and allowed to grow without any treatment. At each phase, samples were taken and analyzed for cell death as in section 4.3.2. The purpose of this experiment was to get a general understanding of cell death levels

throughout the growth phases of untreated PKM cultures. The results show that the proliferative phase cultures show little cell death (low percentage of Annexin V and 7AAD positive while the senescence phase show a higher percentage of cell death, which appears primarily through apoptosis (Figure 4. 3). The majority of PKM in each of these phases remain viable (> 50% even during the senescence phase based on analysis of non-adherent PKM).

4.3.4. Senescence phase primary kidney leukocytes decrease tolerance to H_2O_2

I previously described that the senescence phase CCM has an effect on the proliferative phase PKM cultures by inducing premature senescence, and early cell death, while proliferative phase CCM maintains the culture in the proliferative phase for an extended periods (section 4.3.1 and 4.3.2). Next, I analyzed the effect of the senescence phase CCM on chemical tolerance of the PKM cells. Both proliferative and senescence phase goldfish PKM cells were treated with H_2O_2 as in the materials and methods section (section 2.11). Results from the time course treatment showed that senescence phase PKM cells are more prone to H_2O_2 induced cell death than the proliferative phase PKM culture cells (Figure.4.4). Cell death analysis with flow cytometry showed that H_2O_2 treatment induces cell death primarily through induction of apoptosis (Figure 4.5).

4.3.5. Effect of senescence phase supernatant and rsCSF-1R on goldfish PKM proliferation

4.3.5.1. Goldfish recombinant soluble form of colony stimulating factor-1 receptor (rsCSF-1R) expression

Sf9 insect cell line was transfected using pIB/V5-His TOPO vector, which contains the gene for the goldfish rsCSF-1R. The rsCSF-1R protein was purified from cell culture supernatant and cell lysate according to the procedure in section 2.14). Finally, samples were taken from transfected and untransfected cell lysate as well as transfected and untransfected cell culture supernatants for detection of the expression of rsCSF-1R protein. The analysis was based on bicinchoninic acid (BCA) protein Assay, Coomassie Blue, silver stain, western blot and mass spectrometry. The BCA protein assay measures the total protein concentration in the sample (Fig.4.6A). The silver stains and western blot showed the expected band, which correspond to the molecular weight of rsCSF-1R (about 32 KD) (Fig.4.6B). Finally, the identity of the protein was confirmed by mass spectrometry (Fig.4.7A). All results show that the expression of protein in the supernatant.

4.3.5.2. Senescence phase CCM inhibits cell proliferation in goldfish PKM

The result from the BrdU cell proliferation assay showed that supernatant from senescence phase PKM cultures inhibit cell proliferation while the proliferative and self-CCM maintain the cell proliferation for the extended periods during the course of treatments (Figure 4.8A). Even though the cell proliferation

is significantly different between proliferative and senescence phase CCM treated cells, the overall absorbance reading was low. As the result we also did the manual cell counting using hematocytometer in proliferative and senescence phase CCM treated cells. The result shows that in proliferative phase CCM treated cells the cell number increase from 10,000/100µl to 200,000/100µl in four days while the number of cells in senescence phase CCM increase from 10,000/ 100µl to 95,000/100µl on average (100µl is the volume used per well in 96 microplate) (Table 4.1). On the other hand, results from the treatment of goldfish PKM cultures with rsCSF-1R, showed that rsCSF-1R also inhibits cell proliferation (Fig 4.8B). The anti-rsCSF-1R antibody was added to senescence phase CCM and rsCSF-1R before treating the cells to see if the effect of senescence phase CCM and the rsCSF-1R can be reversed. Treatment of the cultures with the mixture of rsCSF-1R and its antibody as well as the mixture of senescence phase supernatant and antibody against rsCSF-1R showed that addition of anti-rsCSF-1R antibody did not show a significant increase in cell proliferation as expected. The rsCSF-1R and its antibody were mixed and incubated at room temperature for one hour before adding to the cell to allow the binding of the antibody to the rsCSF-1R. The cell proliferation appears to be regained but not fully recovered (Figure 4.9). While the senescence phase CCM decreases the cell proliferation significantly as compared to proliferative phase CCM, the absorbance value from the plate reader was very low in both cases. The inhibition of cell proliferation by senescence phase CCM is not as much as that of the inhibition by purified rsCSF-1R.

4.4 Discussion

As discussed in the previous sections, goldfish PKM cell cultures secrete endogenous soluble growth factors that intrinsically promote cell proliferation (10). These growth-inducing factors are mainly found in proliferative phase supernatants. On the other hand, supernatants from senescence phase cultures are poor inducers of cell proliferation. In this chapter I looked to determine if senescence phase CCM also had growth inhibitory factors that negatively regulate cell proliferation and survival.

My hypothesis was that senescence phase supernatants contain growth inhibitory factors that actively downregulate PKM proliferation and survival. Under conditions of stress and nutrient depletion this may lead to a decreased PKM capacity to proliferate and survive. My results showed that senescence phase supernatants negatively impact goldfish PKM cells proliferation, growth and survival. Senescence phase supernatants induced a premature shift of proliferative phase PKM culture to senescence phase compared to the kinetics observed under normal growth conditions. On the other hand, proliferative phase supernatants lengthen the relative time that a PKM culture will remain in the proliferative phase. From the flow cytometric analysis FSC-H and SSC-H parameters, senescence phase CCM also appeared to induce differentiation of the cells to mature macrophages. Differentiation could be viewed as negative regulation of cell growth as differentiation intimately associated with cessation of cell growth (11,12). As I only used size and internal complexity (granularity) as a

parameter, it is difficult to generalize about this differentiation and need further investigation using other parameters such as gene expression.

Senescence phase supernatants also appeared to induce early cell death in goldfish PKM. My results showed that the proportion of cells entering cell death phenotypes was greater in PKM cultures treated with senescence phase supernatant compared to those treated with proliferative phase supernatant. Further, my results indicate that this cell death was mainly through apoptosis. Analysis of cell death throughout the growth phases of goldfish PKM cultures shows that the percentage of cell death increases in during senescence phase. This suggests that PKM cultures produce growth inhibitory factors into senescence phase supernatants that induce cell death through apoptosis. The difference in the proportion of dead to live cells between treated and untreated PKM cultures may arise from the following possible reasons:

1. Only non-adherent cells were used because of the difficulties in detaching adherent goldfish PKM without damaging the cells that interfere with the analysis while the untreated cultures have both adherent and non-adherent cells.

2. As the cells in the culture continue to grow, they fill the available space in the culture (confluence). As the result nutrient depletion occurs in the media, apoptotic and necrotic debris accumulated, high density induces cell-to-cell contact stimulation for differentiation and cell cycle arrest. In this experiment, the cells were split and treated with different phases of supernatant and some new

media was also added to the cultures. These factors provide additional nutrients for the cells to survive and/or proliferate.

3. Senescence leads cells to death but can these may remain viable for some time though it decreases proliferation (14,15). One of the different factors that induce cellular senescence is the environmental stress (due to the cumulative effect of different factors). Therefore, the senescence phase supernatant can decrease cell proliferation instead of inducing more cells death. In summary, the senescence cells show features like extended cytoplasm, flattened, and larger size, increased response to contact inhibition, and losing its original shape (13). Therefore, even though the proportion of dead to live cells are low, the senescence CCM treated cells show the aforementioned features that indicates the cells are entered to senescence phase.

Senescence phase supernatant also reduces the tolerance of the PKM cells to H_2O_2 induced cell death. Activated macrophages and monocytes produce different lytic factors and enzymes like reactive oxygen species (NO and H_2O_2) during phagocytosis and inflammatory response (14). During phagocytosis, they ingest particles in small membrane vesicles. These membrane vesicles fuse to lysosomes and form phagolysosomes. These contain reactive oxygen species, which digest and kills pathogens (15,16). Therefore, these reactive oxygen species are important in antimicrobial defense, and under normal circumstances, the phagocytes have a mechanism to turn off the chemicals such as using catalase or by conversion of one reactive oxygen species to other reactive species (17,18) to prevent the excessive generation that may be toxic and affect the cell itself. This

has an impact on the inflammatory reactions (19) and can affect the efficiency of macrophages in removing pathogens. Senescence phase goldfish PKM are more prone to H_2O_2 toxicity than proliferative phase and this toxicity appears to induce apoptosis. Senescence, therefore, can potentially affect the function of PKM in immune response by reducing the cells tolerance to oxidative stresses. In other words, my results highlight the potential implications of intrinsic cell growth regulatory mechanisms exhibited by PKM *in vitro*. Among others, induction of apoptotic cellular death will have an anti-inflammatory impact within an inflammatory site (20, 21). On the one hand, this could contribute to the resolution of inflammation through a shift towards homeostatic maintenance or it could facilitate pathogen expansion/disease progression in those cases where the inflammatory process may have shown partial effectiveness against infiltrating pathogens (22).

Senescence phase supernatants also negatively impact cell proliferation. PKM incubated with senescence phase supernatants show lower rate of proliferation when compared to cells incubated with proliferative phase supernatants. Treatment of PKM with the mixture of senescence phase supernatants and antibody against rsCSF-1R partially recapitulated the results observed with proliferative phase supernatants. The results show that cell proliferation is not recovered fully, which indicates that the effect of cell proliferation may not be only from the effect of sCSF-1R. There could be other factors that contribute for the negative regulation of the cells. As the CCM is a crude source of cellular factors, it may contain different factors that are secreted

or released to the media from the cells and inhibits the cell proliferation (23,24). Longer incubation period of the antibody with the senescence phase supernatant before adding to the cells may also help to fish-out all the possible sCSF-1R from the supernatant and increase the cell proliferation. The difficulty in this case is that since the antibody against sCSF-1R can also bind to membrane bound receptors and decreases the cell proliferation (data not shown) and since it is complex to know the actual concentration of sCSF-1R in the supernatant, it is difficult to add the same concentration of the antibody to the supernatant.

Recombinant soluble form of CSF-1R also inhibits the cell proliferation (9). The purified rsCSF-1R affects the cell proliferation more than the senescence supernatant. From this, we can infer that the concentration of native sCSF-1R in the supernatants appears very low. Overall, the senescence phase supernatants contain growth inhibitory factors that negatively regulate the PKM growth, proliferation and survival. Of these negative regulators, soluble CSF-1R has a significant role as inferred from the comparison of the effect of senescence CCM and rsCSF-1R on PKM cell proliferation.

Table 4.1. Goldfish PKM proliferation determined by counting cell numbers. After treating with proliferative and senescence phase CCM. Goldfish PKM cells were subdivided and treated with proliferative and senescence phase CCM (10,000 cells/well in 96 well microplates) for four days and the cell number was counted by hematocytometer to determine the cell proliferation.

Sample	Proliferative CCM	Senescence CCM
Fish 1	1.79×10^5	1.01×10^5
Fish 2	2.75×10^5	1.20×10^5
Fish 3	1.02×10^5	7.8×10^4

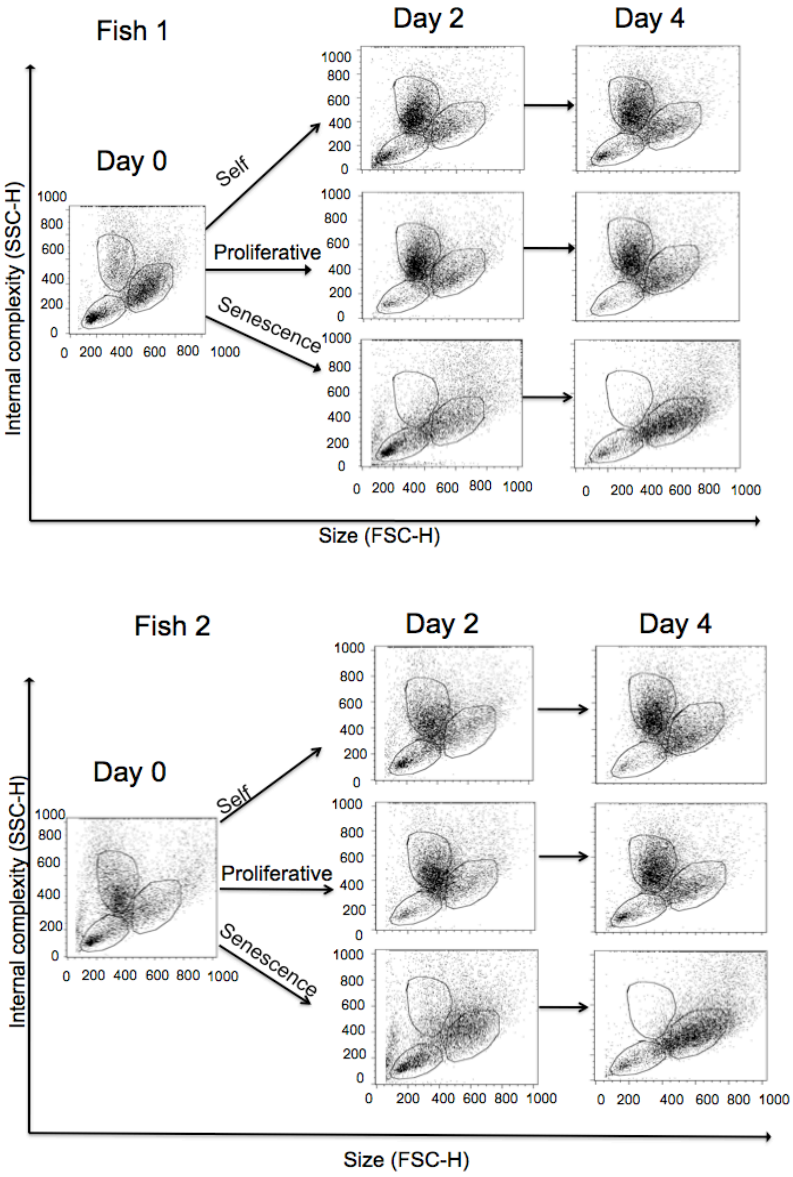

Figure 4.1. Effect of senescence phase CCM on proliferative phase PKM cultures analyzed by flow cytometery. Goldfish PKM cultures were grown for 6 days. Then non-adherent cells were subdivided in to three flasks for the treatment with its own CCM (self), proliferative phase CCM and senescence phase CCM for 4 days and then analyzed with flow cytometery using forward scatter for size (FSC-H) and side scatter for granularity (SSC-H) on day 2 and 4 post treatment. Two representative figures are shown (n=4).

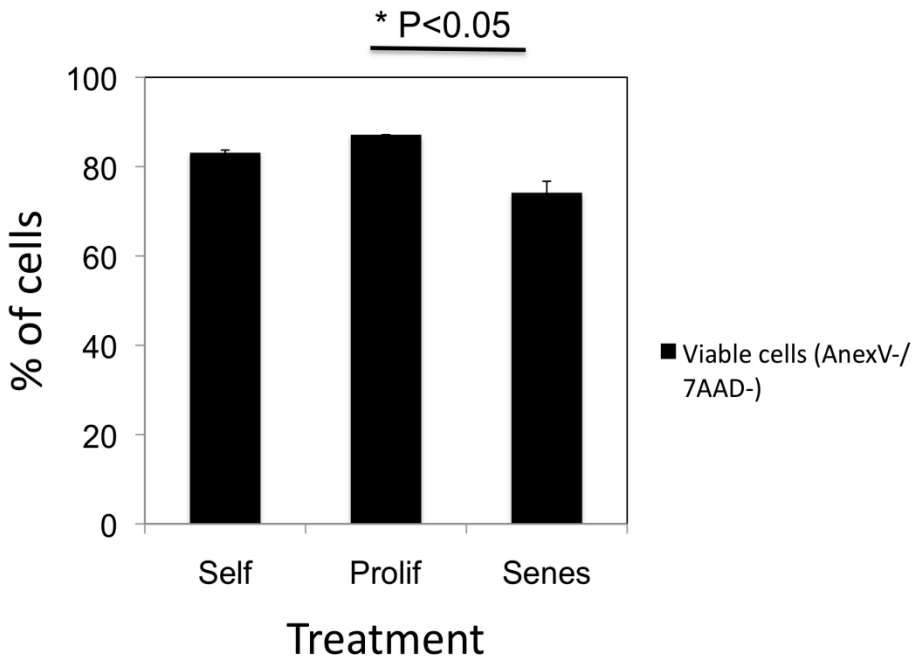

Figure 4.2. Analysis of cellular death in goldfish PKM treated with different phases of CCM. Proliferative phase PKM were subdivided and treated with different phases of CCM as in Fig.4.1. Then cells were centrifuged and the pellet was washed twice with 1×$PBS^{-/-}$, resuspeneded in Annexin V buffer to the final concentration of 1×10^6 cells/ml and analyzed by flow cytometry. The total percentage of live cells (Annexin V-/7AAD- cells) was compared between proliferative and senescence phase CCM treatment. The error bars show the ±SEM for six fish and the "*" shows the statistical significance at $P<0.05$.

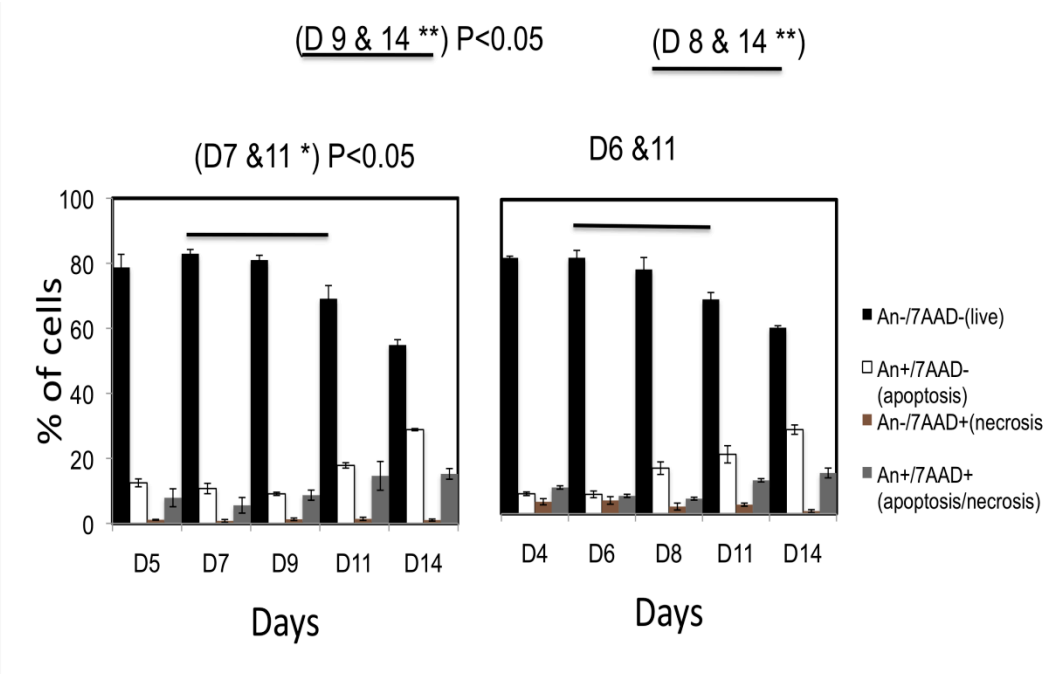

Figure 4.3. Cell death analysis in untreated PKM through out the growth phases of the cultures. Goldfish PKM were cultured based on our conventional procedures with proliferative phase CCM and allowed to develop through a 14-day *in vitro* growth period. Sample cells from different phases of each culture were analyzed for cell death as in Figure.4.2. Day 0- 4 corresponds to the late lag phase. Day 6-9 corresponds to the proliferative phase, and Day 11-14 to senescence phase. The error bars show the ±SEM of three fish for each figures and figures from two independent experiments are show. The total percentage of cell death is compared between proliferative and senescence phase PKM and the statistical significance is shown by * between early proliferative and early senescence phases while ** is between late proliferative and late senescence phases at $P<0.05$.

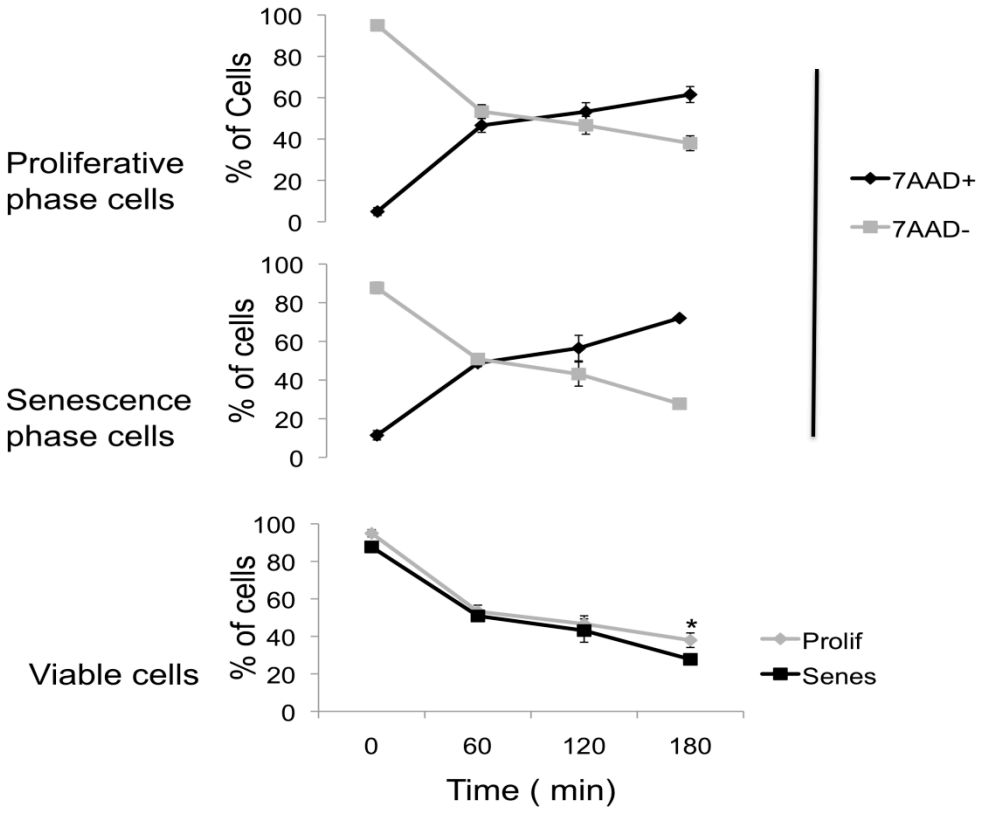

Figure 4.4. Comparing the chemical sensitivity between proliferative and senescence phase PKM cells. 1) 7AAD+ represent cells, which are stained by 7AAD and considered to be dead. 2) 7AAD- are cells, which exclude 7AAD, and presumed to be live cells. Cells from proliferative and senescence phase cultures of the same fish were treated with H_2O_2 (final concentration of 0.088 mM) for 0, 60,120 and 180 minutes and stained with 7AAD and finally analyzed by flow cytometery. In both cases H_2O_2 was added to the media instead of adding directly to the cell to reduce uneven contact toxicity and in all cases the same volume of media and cells were used by pre diluting the cells to required concentration. The error bars show ±SEM from four fish. The "*" shows the statistical significance at $P<0.05$.

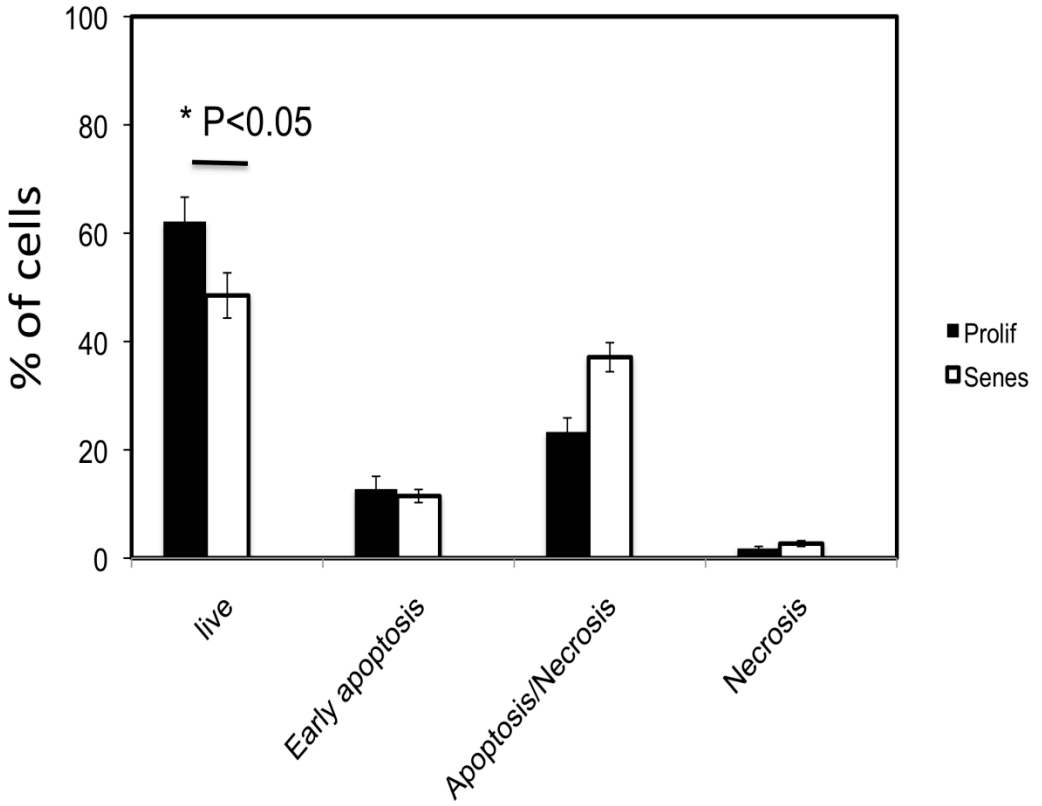

Figure 4.5. Senescence phase CCM increase susceptibility of PKM to H_2O_2 induced cellular death. Both proliferative and senescence phase PKM cells were treated with H_2O_2 (final concentration of 0.0882 μM) for 10 minutes. Treated cells were centrifuged, pellet washed twice with $1xPBS^{-/-}$ and resuspended in the Annexin V buffer and finally the cell death was analyzed by flow cytometery. 1) Prolif = proliferative phase PKM cells.2) Senes = senescence phase PKM cells. The total percentage of dead cells (Annexin V positive, 7AAD positive and Annexin V/7AAD positive) is compared between proliferative and senescence phase PKM cells. The error bars show the ±SEM for four fish and the * shows the statistical significance at $P<0.05$.

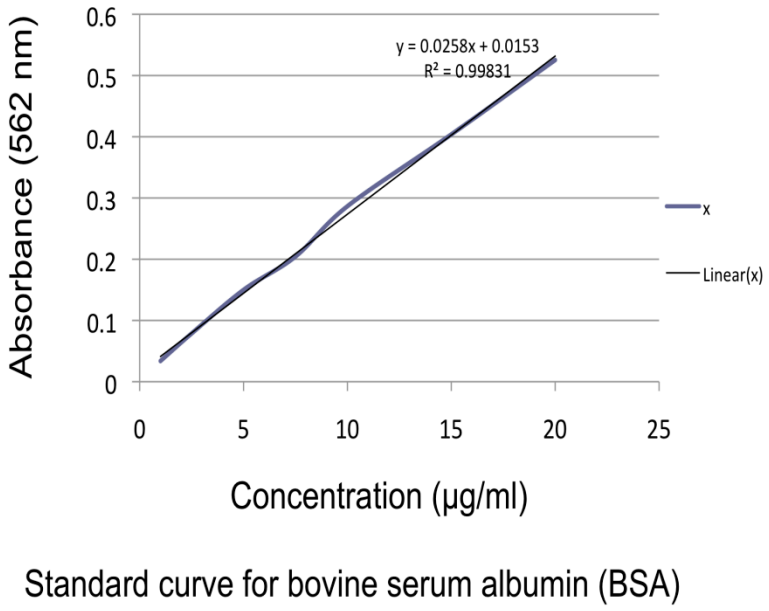

Figure 4.6A. Expression and identification of sCSF-1R recombinant protein produced in sf9 insect cell line. The concentration of purified protein from insect cell supernatant was determined by BCA protein assay using standard curve developed with known concentration of BSA. The BSA (Thermo Scientific, Rockford, IL. U.S.A) was diluted to different concentration according to the manufacturer's specification and the absorbance of each dilution was taken. Then the protein sample was also diluted and absorbance was taken. Based on the absorbance of diluted protein and BSA standard curve, the concentration of diluted protein sample was determined. Using the concentration of diluted sample and dilution factor, the concentration of original protein concentration was determined and appears to be (550 µg/ml).

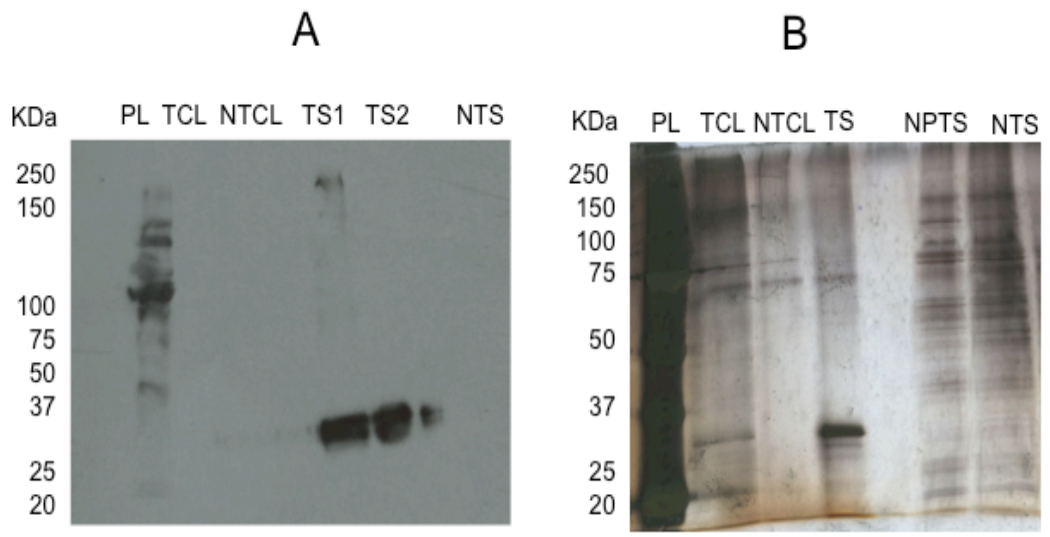

Figure 4.6B. Western Blot. The protein was separated on 10 % SDS-PAGE. A) Western blot. The protein was separated on 10% SDS-PAGE, and the separated protein band was blotted to transfer on to nitrocellulose membrane. The membrane was then blocked by 5% skim milk in PBS for one hour and incubated overnight with primary antibody (Rabbit polyclonal antibody to 6x His tag according to the manufacturers specification, with the dilution of 1:500, (BIO-RAD). After washing the membrane three times with PBS, the membrane was incubated with secondary antibody according to manufacturer's specification (Goat anti rabbit IgG-HRP antibody, R&D), with the dilution of 1:1000. Finally, the developing substrate was added in equal volume to the membrane and the color development was detected using ECL detection kit (GE Healthcare) in the dark room according to the manufacturer's specification. The two bands (TS 1 and TS2) are from the two batches culture of supernatant. B) Silver stain after the protein was separated on SDS-PAGE.

PL=protein ladder

TCL=transfected cell lysate

NTC=non transfected cell lysate

TS=purified supernatant from transfected cell

NPST=non purififed supernatant from transfected cell

NTS=non transfected cell supernatant

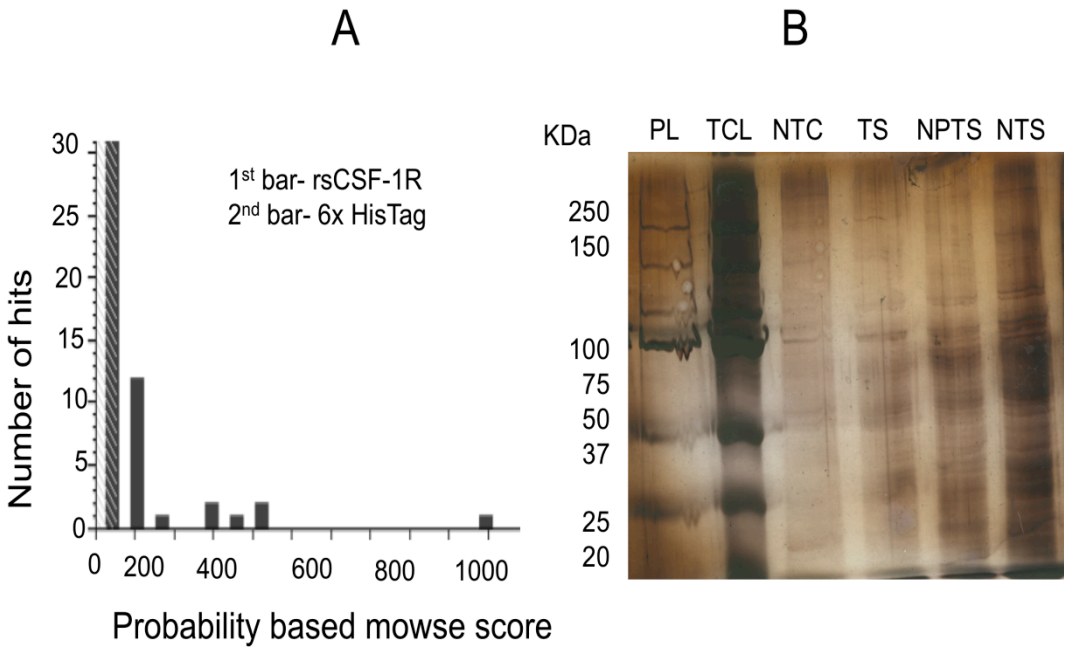

Figure 4.7. Identification of rsCSF-1R protein by Mass spectrometer. Purified protein from transfected cell supernatant was separated using 10% SDS-PAGE. The gel then stained by Coomasie blue to see the band for protein of interest. The band was analyzed by mass spectrometry using proteomic software, Mascot (Version2.2, Matrix Science) (A). The different bars are representing proteins in the solution and the percentage of hits is considered for the density of protein in the solution. The first two bars are representing rsCSF-1R protein and artificial construct (His-tag) protein. Silver stains to visualize the protein band of interest in the purified solution (B). Abbreviations are as per Figure 4.6b.

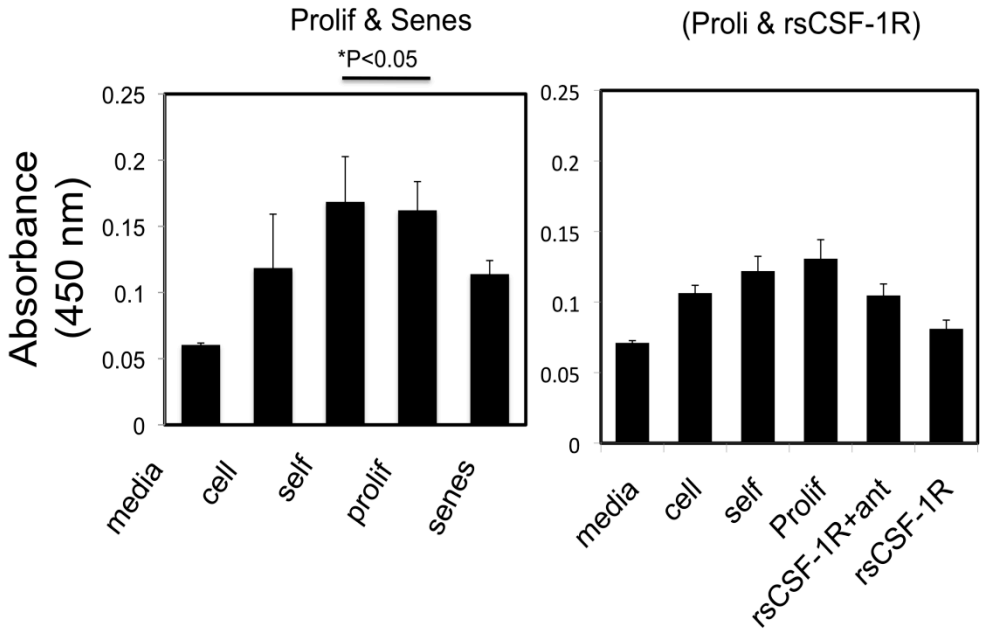

Figure 4.8. Effects of senescence phase CCM and rsCSF-1R on goldfish PKM proliferation. PKM cells (50,000 cells/well) were cultured in 96 well microplates with self-CCM, proliferative CCM and senescence CCM (A) or Self-CCM, proliferative CCM, rsCSF1-R (10 ng/ml), the mixture of rsCSF-1R (10 ng/ml) and antibody against rsCSF-1R (1:500 dilution) (B) for two days and then BrdU labeling solution (final concentration of 10μM) was added and incubated for 24 more hours. Finally The BrdU incorporation was analyzed as of manufacturer's specification (Roche). Comparison was between senescence phase and proliferative phase CCM (A) and between proliferative CCM and rsCSF-1R (B). The difference at $p<0.05$ is considered to be statistically significant (*) using unpaired, two tailed t-test (Graphpad software). The error bars show ± SEM for three fish each with triplicate experiment (n=3).

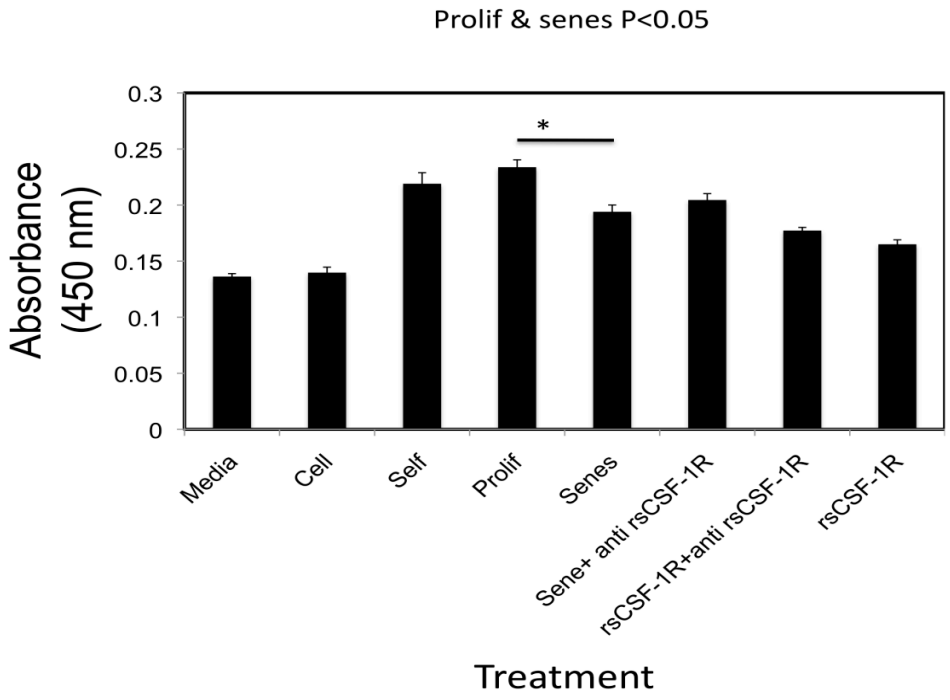

Figure 4.9. Effects of anti-rsCSF-1R antibody on the restoration of the PKM proliferation after inhibiting by the senescence phase CCM and rsCSF-1R. PKM cells (50,000 cells/well) were cultured in 96 well microplates as in Figure 4.8. The treatment of the mixture of senescence phase CCM and antibody against sCSF-1R (1:500 dilution) (senes+anti) as well as the mixture of rsCSF-1R (10 ng/ml) and antibody against rsCSF-1R (1:500 dilution) (rsCSF-1R+anti) were added to see if the inhibition of cell proliferation by senescence phase CCM and rsCSF-1R was reversed. The comparison is between the proliferative phase CCM, senescence phase CCM and rsCSF-1R. The difference between all the three treatments is statistically significant ($p<0.05$) using two-tailed unpaired t-test. The error bars show ± SEM for five fish and each experiment was done in triplicates. The star shows significant different between the proliferative phase and senescence phase supernatant.

4.5. References

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CHAPTER VI: GENERAL DISCUSSION

5.1. Overview of the findings

The work outlined in this thesis indicates that senescence phase supernatants negatively impact the growth, survival and proliferation of goldfish PKM cells. I based my analyses on factors associated with cell survival, culture morphology, cell proliferation, and the cells` tolerance to H_2O_2 induced chemical toxicity. The proliferative and senescence phase of goldfish PKM have distinct features in their morphology, flow cytometric profile, cell composition and production of growth factors as well as the rate of cell proliferation (1, 2). The study of goldfish PKM cultures treated with senescence phase CCM show that senescence phase supernatants appears to promote early senescence, which is an indication that the senescence phase supernatant is not only poor growth inducer but also contains growth inhibitory factors that induce cellular senescence (3,4). Therefore, the responses of PKM to senescence phase supernatants by entering to senescence is potentially driven by soluble inhibitors in the senescence phase supernatant that inhibit the cell proliferation and survival. It can also be associated with the response of the cells to avoid the overall stress from the senescence phase supernatant due to nutrient depletion, and accumulation of apoptotic, and necrotic bodies. As the CCM is crude supernatant that contains hundreds of protein (5), there may be different growth inhibitory factors in the supernatant that affect the growth and survival of the PKM hence needs furthers study to identify the potential inhibitory factors in the senescence supernatant that can negatively affect the cell proliferation and survival. Furthermore, these effects can also be

due to withdrawal of growth inducing factors that can potentially lead the cells to senescence (5-9).

The effect of senescence phase supernatants on the macrophage growth pattern also looked to see whether the phenotypic changes in the cell cultures were due to change in the media composition or as the result of treatment with senescence supernatant. The main objective of this analysis was to see if the incubation period and temperature affect the media composition, which intern affect the cell growth. For example, glutamine is an amino acid, which is important in the animal cell culture serving as an alternative energy source, protein and DNA synthesis but it is unstable and can be decomposed through time (10, 11). As the result, the temperature and prolonged incubation may affect the glutamine stability, which can also affect the growth of the cells (12). My results show that the pre-incubation of the media alone does not affect the cell growth indicating that the pre-incubation did not affect the glutamine stability or the goldfish PKM cells are not affected by decomposition of glutamine. On the other hand, it also shows that the senescence phase supernatant contains factors that affect the growth pattern and phenotypes of the cultures (13).

Senescence phase supernatants also induce early cell death through apoptosis in goldfish PKM as opposing to proliferative phase supernatant, which maintain the cells in proliferative phase for a longer periods suggesting that senescence phase supernatant negatively regulates the survival of PKM. Apoptosis plays an important role in homeostasis. Cells also execute apoptosis during environmental stresses (7, 14). Senescence phase supernatants induce cell

death potentially due to the growth inhibitory factors in the senescence supernatant that down regulate the cell survival and/or the cells may be undergoing apoptosis because of environmental stress due to accumulation of cellular debris and metabolic wastes in the senescence phase supernatants (15).

Further analysis indicates that senescence phase supernatant also decrease the ability of goldfish PKM cells to resist environmental stress. Results from the treatment of goldfish PKM cells with H_2O_2, show that senescence phase cells are more prone to H_2O_2 induced cell death. On the other hand, the proliferative phase goldfish PKM cells are more resistant to oxidative stress. The loss of tolerance to oxidative stress in senescence phase cells has an impact on the efficiency of macrophages during inflammatory response as macrophages produce reactive oxygen species during inflammatory reactions (16-18). Oxidative stress may come either from the intracellular or extracellular and have a significant effect on the function of macrophages during inflammatory response and phagocytosis (19, 20). Consequently, senescence phase supernatant appears to affect the function of the macrophages.

Senescence phase supernatants also inhibit goldfish PKM proliferation. There are different extracellular and intracellular factors that reduce signal cascade and inhibit the cell proliferation (18, 21, 22). In goldfish PKM, one of these factors is soluble form of CSF-1R that prevents the binding of CSF-1 to its membrane bound receptor, consequently inhibits cell proliferation. The rsCSF-1R shows more inhibition in goldfish PKM proliferation than the senescence phase supernatant. This could be because of the low concentration of native soluble

form of CSF-1R in the supernatant. Some other factors may also exist in the senescence phase supernatant that have a negative impact the PKM proliferation and survival besides sCSF-1R (23-26) and need further study to identify potential growth inhibitory factors. Overall, the discovery of soluble form of CSF-1R in senescence phase supernatants, the inhibition of cell proliferation by recombinant soluble CSF-1R (26) as well as the impact of senescence phase supernatant on goldfish PKM growth, proliferation and survival is an indication that the native soluble form of CSF-1R contribute to the negative regulation of goldfish PKM. Further understanding of the evolutionary conservation of mechanisms that regulate macrophage function and development is important. This work provides additional insights into the understanding of the negative regulation of macrophage survival and proliferation.

5.2. Future studies

Suppression of the immune response is as important as activation or up-regulation of the immune system. This project focused on the endogenous products that PKM use to down regulates their development and survival. I showed that senescence phase supernatants contain growth inhibitory factors that negatively impact the PKM proliferation and survival. From my analysis of the impact of rsCSF-1R and senescence phase supernatant, the sCSF-1R contributes in the negative regulation of macrophage survival and proliferation. I believe that additional investigations on the role and extent of the sCSF-1R in macrophage self-regulation are warranted. Specifically, the following areas may present potentially fruitful lines of study:

1. Functional analysis of PKM as they progress through proliferative and senescence phases of development. This may provide insights into the functional heterogeneity exhibited by macrophage populations in vivo..

2. Analysis of other soluble factors contained within senescence phase supernatants. In particular, it will be interesting the level of pleiotropy and redundancy exhibited by factors derived from macrophages of a lower vertebrate species.

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Appendix A

Cell culture status

Date:_____

Culture day:_____

PKM: _____

CCM source: _____

Media color:_____

Flow analysis done: Yes No

Overall cell density (low power):_____

Bacterial/Fungus contamination? Yes No

Debris? Yes No

Amount: low, med, high

Size: dot med, large

Non-adherent Population:

Density: low, med, high

Average Morphology: nice round

Activated/elongated

Other

Opaque/vacuolation/irregular shape (Bad)

Mostly highly refractile (Good)

Adherent Population:

% Floor covered: ~ ____ %

Ave. % of cells with vacuolation: not **vacuolated**_____

Ave density of vacuoles/vacuolated cell: low, med, high

Ave. Morphology:

Irregular shaped singlets

Density: low, med, high

Fried eggs: Small

Density: low, med, high

Medium

Density: low, med, high

Giant-multinucleated? Yes No

Density: low, med, high

Clumps? Yes, No

Amount: low, med, high

Size: ≤ 10 cells ~10-30 cells ≥ 30 cells/ clump

Islands? Yes, No

Amount: low, med, high

Size: ≤ 10 cells ~10-50 cells ≥ 50 cells/ island

Other comments: