INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

University of Alberta

GENE EXPRESSION IN BLASTOCYSTS AND THE ENDOMETRIUM DURING PORCINE PERI-IMPLANTATION DEVELOPMENT

by

Linghuo Jiang ©

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

in

Animal Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall, 1999

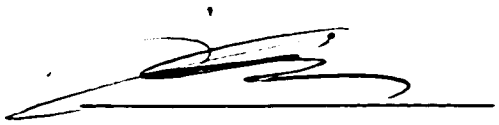
UNIVERSITY OF ALBERTA

Library Release Form

Name of Author:	Linghuo Jiang
Title of Thesis:	Gene Expression in Blastocysts and the Endometrium during Porcine Peri-implantation Development
Degree:	Doctor of Philosophy
Year This Degree Granted:	1999

Permission is hereby granted to the University of Alberta library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.



407 RH, Michener Park
Edmonton, Alberta
Canada T6H 4M5

Date: Oct. 5, 1999

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **GENE EXPRESSION IN BLASTOCYSTS AND THE ENDOMETRIUM DURING PORCINE PERI-IMPLANTATION DEVELOPMENT** submitted by Linghuo Jiang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science.

Dr. Walter T. Dixon (Supervisor)

Dr. George R. Foxcroft

Dr. Larry J. Guilbert

Dr. Robert J. Christopherson

Dr. Gilbert A. Schultz

Date October 4, 1999

Dedicated to

my wife, my daughter,

my parents, and my parents-in-law

for

their constant support during the course of this study.

ABSTRACT

Macrophage colony-stimulating factor (CSF-1) was found to be the major factor, in conditioned media of Jag-1 cells, responsible for stimulating porcine macrophage proliferation *in vitro*. A porcine CSF-1 cDNA was isolated and characterized from Jag-1 cells. The CSF-1 gene was found to be present as a single copy in the porcine genome. Expression of CSF-1 transcripts was up regulated in both blastocyst and endometrial tissues during the peri-implantation period in the pig, with higher levels in endometrial tissues than blastocyst tissues. Similarly, higher levels of c-fms (CSF-1 receptor) transcripts were detected in endometrial tissues than in blastocysts. Our data suggest that CSF-1 might exert both paracrine and autocrine effects on porcine pre-implantation embryonic development.

Expression of matrix metalloproteinase (MMP) transcripts and their regulators was examined in pregnant gilts during the peri-implantation period. In blastocysts, MMP-2, MMP-14 and TIMP-2 increased, while MMP-9 was undetectable. MMP-8, TIMP-1 and TIMP-3 transcripts also increased, whereas uPA exhibited a biphasic pattern of expression. In the endometrium, expression of MMP-1, MMP-2, MMP-8, MMP-9, MMP-11, MMP-14, uPA, TIMP-1, TIMP-2 and TIMP-3 were all observed, but not MMP-7 and MMP-13. ProMMP-2 and TIMP-2, but not proMMP-9, were detected in uterine flushings. A lack of MMP-9 expression in blastocysts might explain the non-invasive implantation seen in pigs. Co-expression of MMP-2, TIMP-2 and MMP-14 in blastocysts might contribute to extraembryonic mesoderm cell migration.

Expression of integrin subunit (α1, α2, α3, α4, α5, αv, β1 and β3) transcripts was also determined during early gestation in pigs. In endometrial tissues, constant

expression of αv, β1 and β3 was detected, whereas α1, α2, α3 and α5 appeared to be developmentally regulated. Expression of α2, α3, αv, β1 and β3 exhibited similar patterns and was developmentally regulated in blastocysts, with high levels in 1-2 mm spherical blastocysts, low levels in 5-10 mm spherical blastocysts and variable levels in elongated blastocysts. In Day-28 placental tissue, moderate expression of α1, α3, αv and β3 and weak expression of α2 and β1 was observed. Jag-1 cells expressed high levels of α1, α2, α3, αv and β3 and low levels of α5 and β1. Our findings support an important role for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig. In summary, the present study has identified and characterized the expression of some important protein factors, in blastocysts and the endometrium, that are hypothesized to play critical roles in conceptus development during early gestation in the pig.

ACKNOWLEDGEMENTS

First I would like to thank Dr. W.T. Dixon for giving me this opportunity to complete my Ph.D. study in his lab and fulfill my dream in the area of research I have been interested in. I am especially grateful for his excellent supervision as well as his emotional support and encouragement at the worst of times during the course of this study. Because of his kindness and wisdom, my life as a Ph.D. student has turned to be an enjoyable learning experience. Special thanks are also given to Dr. G. R. Foxcroft for his guidance and excellent instruction in the area of reproductive biology. My sincere appreciation also goes to Dr. L.J. Guilbert for his supportive service on my Supervisory Committee. Furthermore, I would like to thank Renate Meuser and Joan Turchinsky for their generous technical support all the way through this study. Special thanks go to Susan Novak for her help in collection of uterine and embryonic tissue samples, and to Dr. Denis Balcerzak, Richard Y.Z. Chai, Dr. Brian Treacy and Colin Strauss for their valuable advice and stimulating discussion. In addition, I want to thank all members of Dr. G.R. Foxcroft's research group and all members at the Molecular Biology and Biotechnology center for their friendship and the fun times we had in the laboratory. Finally, this study would not be possible without the support of the 1995/1996 VP Academic (Ms. Monika Lozinska) of the GSA, the previous departmental Graduate Committee Chair (Dr. John Feddes), and two of my previous supervisory committee members (Drs. Kenneth Roy and Andrew Keddie). Of course, I should also thank Dr. Chuji Hiruki for his generous two-and-half-year financial support from NSERC for my technical and ESL training in his laboratory.

TABLE OF CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

APPENDIX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CSF-1 or M-CSF	Macrophage colony-stimulating factor
c-fms	Receptor for macrophage colony-stimulating factor
CT	Cytotrophoblast
ECM	Extracellular matrix
EGF	Epidermal growth factor
FCS	Fetal calf serum
IFNγ	Interferon-γ
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HBGF	Heparin binding EGF-like growth factor
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IL	Interleukin
INF	Interferon
Jag-1	Porcine trophoblast cell line
mRNA	Messenger ribonucleic acid
MGF	Macrophage growth factor
MMP	Matrix metalloproteinases
Mt-MMP	Membrane type-MMP
PDGF	Platelet-derived growth factor
PBS	Phosphate buffered saline
RP-FPLC	Reverse phase-fast protein liquid chromatography
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ST	Syncytiotrophoblast
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

CHAPTER 1 LITERATURE REVIEW

INTRODUCTION

During this century, increases in reproductive efficiency of livestock have mainly resulted from improved nutrition and management practices by increasing the frequency of complete reproductive cycles. Study of embryonic mortality in domestic species has become an increasing interest of research in recent years. Regardless of species, prolificacy is primarily regulated by number of eggs ovulated and fertilized, embryonic/fetal survival and neonatal survival (Bazer, 1994a). Reproductive performance in swine could be largely limited by ovulation rate and prenatal embryonic mortality, because fertilization rate in swine does not seem to limit reproductive efficiency (Van Der Lende et al., 1994; Ford and Youngs, 1993; Perry and Rowlands, 1962). However, inseminations performed too early or too late relative to ovulation reduced fertilization rates in sows (Kemp and Soede, 1997). Ovulation rate could be improved by increasing energy intake during late diestrus and proestrus, injection of exogenous gonadotropins, or by genetic selection (Wrathall, 1971).

Embryonic/fetal mortality in pigs may reduce potential litter size by 40 to 50% (Bazer, 1994a). Differences between parities may exist with prenatal mortality ranging from about 30% at parity 1 to 50% or more at higher parities (Foxcroft, 1997). It has generally been accepted that about 75% of the total prenatal loss occurs during the first 30 days of gestation (Ford and Youngs, 1993; Pope and First, 1985). However, even with high ovulation rates, lower embryonic mortality before Day 28 of pregnancy may occur in modern genotypes, resulting in loss of up to 50% of surviving embryos in the post-implantation period (Foxcroft, 1997). The underlying causes of embryonic mortality are

complex and could be derived from chromosomal aberrations in the embryo (Bishop, 1961; Day and Polge, 1968), genetic traits (Bishop, 1961; Perry and Rowlands, 1962), environmental factors (Wildt et al., 1975), nutrition (Foxcroft, 1997) and intrauterine infections (Scofield et al., 1974). However, a difference exists in embryonic mortality between Days 25 to 30 (30%) and Day 12 (5 to 10%), which suggests that considerable losses occur from Days 13 to 20 (Pope, 1994). This period of high embryonic mortality is coincident with blastocyst elongation and attachment in the pig (Perry and Rowlands, 1962), processes regulated *via* steroid and protein factors secreted from the endometrium and the embryo. Therefore, elucidating the mechanisms regulating blastocyst-endometrial interactions during blastocyst elongation and attachment will lend insight to aberrant processes of embryogenesis that may contribute to embryonic death in this important domestic animal.

In this literature review, embryonic development in early pregnancy in the pig will be briefly documented, followed by a discussion of the maternal recognition of pregnancy that is a critical step for prolonged maintenance of functional corpora lutea. Emphasis will be given to the possible mechanisms regulating blastocyst-endometrial interactions during blastocyst elongation and attachment in the pig. Relevant information from other mammalian species will be cited as needed.

EARLY EMBRYONIC DEVELOPMENT IN THE PIG

A. Cleavage

As in other mammalian species, the porcine egg is the largest cell, with the highest ratio of cytoplasmic to nuclear content in the female body, although because of the absence of yolk materials it is smaller than the eggs of other vertebrates such as fish,

amphibians, reptiles and birds. Fertilization activates a porcine egg to complete the second meiotic division and form a female pronucleus in the cytoplasm, which fuses with a male pronucleus derived from a penetrated sperm to produce a zygote (one-cell embryo). Immediately after the formation of the zygote, the fertilized egg undergoes several successive mitotic divisions, or cleavage, inside the zona pellucida without any increase in total mass (McLaren, 1972). The cells formed in the process of cleavage are known as blastomeres. In the pig, fertilization takes place in the ampulla of the oviduct, and the first cleavage normally occurs within 18 hr after ovulation, or around Day 2 of pregnancy (Day 0 is the onset of estrus) (Figure 1.1). The 2-cell stage of the embryo lasts for 6-8 hr, and the 4-cell stage takes 20-24 hr (Hunter, 1974; Flint, 1981). Porcine embryos move from the oviducts and into the uterine horns at about the 4-cell stage, i.e. Day 3 of pregnancy (Hunter, 1974; Bazer et al., 1982). Thereafter, further cleavage divisions follow until embryos reach the 16- or 32-cell stage in the uterus. Since the blastomeres are contained within the zona pellucida throughout the period of cleavage, the resultant group of blastomeres appears much like a solid ball of cells or a mulberry (Patten, 1948), and embryos at these stages are described as morulae.

B. Compaction and Blastocyst Formation

During late cleavage stages of embryos in the pig, blastomeres begin a process of compaction, by which loosely attached blastomeres become tightly packed together and joined by intercellular junctions. The first signs of compaction are seen as early as the 8-cell stage, or at Day 4 of pregnancy (Hunter, 1974). Compaction initiates the process of epithelial differentiation and, consequently, results in the formation of the ectoderm from the outer cell layer. The group of cells inside the ectoderm remains

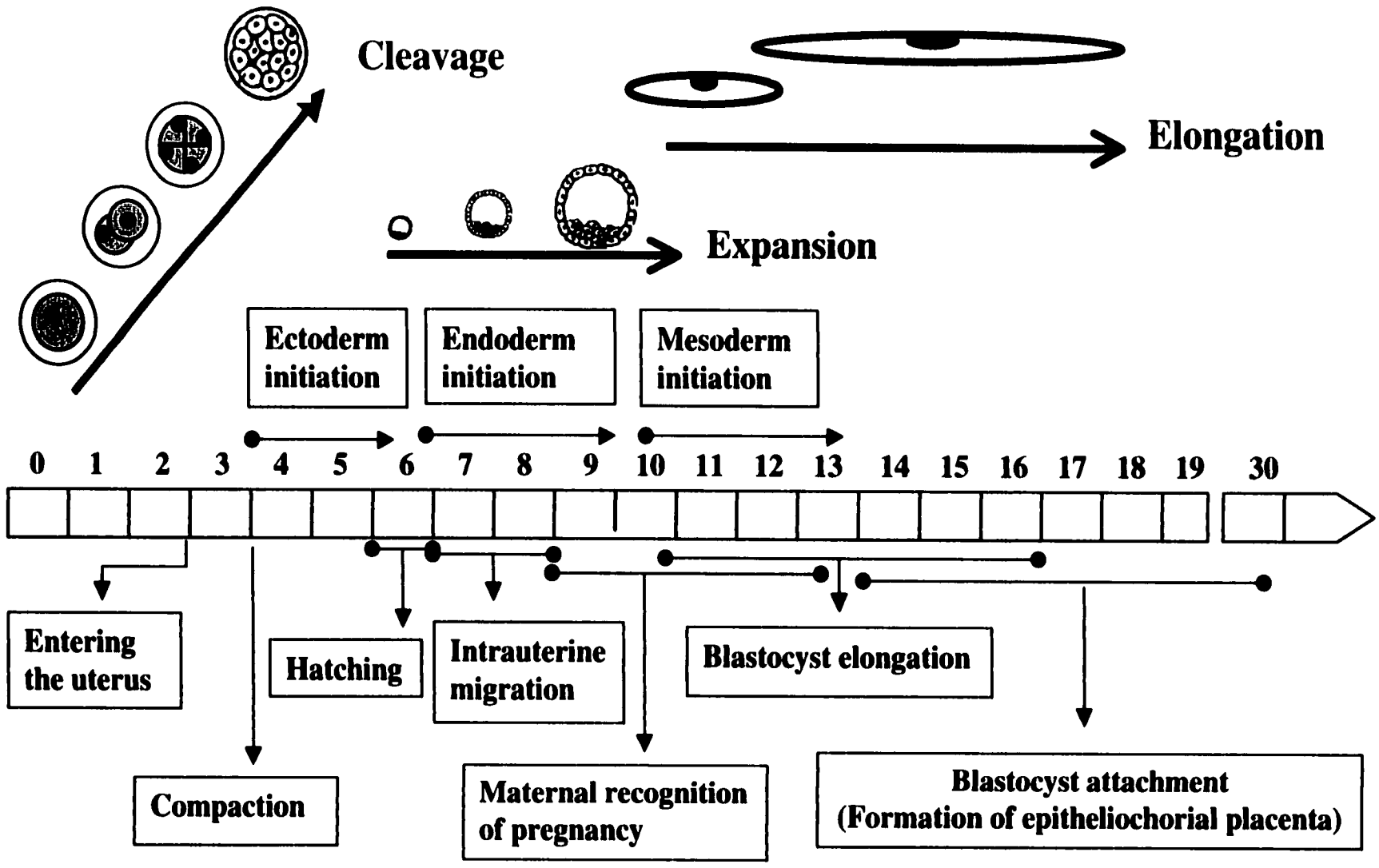


Figure 1.1 Early embryo development in the pig.

undifferentiated and is called the inner cell mass (ICM). Due to the inward fluid transport by the ectoderm cells, a fluid-filled cavity (blastocoele) appears within the morula, which marks the formation of the blastocyst by Day 5 (Papaioannou and Ebert, 1988). Once the blastocoele is established, it enlarges rapidly. Subsequently, blastocyst hatching from the zona pellucida occurs at Day 6-7 (Hunter, 1977), which may be caused by embryonic enzymes, mechanical mechanisms or uterine factors (Stone et al., 1986; Stroband and Van der Lende, 1990). The newly hatched blastocyst is about 0.2 mm in diameter (Geisert et al., 1982a).

C. Formation of Germ Layers and Extraembryonic Membranes

The first germ layer formed during embryonic development is the ectoderm, which consists of the embryonic **ectoderm** and the extraembryonic **trophectoderm** or **trophoblast**. As the hatched blastocyst expands, the ICM cells proliferate rapidly *in situ*. Some of the ICM celis form an outgrowth along the inner surface of the trophectoderm into the blastocoele (Patten, 1948; Geisert et al., 1982b), and subsequently form a complete layer of cells called the extraembryonic **endoderm** - the second germ layer (Patten, 1948; Barends et al., 1989). While the **endoderm** is being established, the embryonic ectodermal cells overlying the ICM degenerate; simultaneously, the ICM cells proliferate further and become aggregated into a disk-shaped and thickened area, which is sharply distinct from the adjoining trophectoderm. This area is called the **embryonic disc** (Patten, 1948; Geisert et al., 1982b).

Immediately after the endoderm has been established as a definite layer, the blastocyst undergoes a striking elongation from a spherical shape of about 10 mm in diameter to a filamentous shape of up to 100 cm in length (Anderson, 1978; Geisert et al.,

1982a; Stroband & Van der Lende, 1990). At about the time the blasotcyst begins elongation, a local differentiation occurs at one part of the margin of the embryonic disk, and the cells in this region undergo proliferation inward to the endoderm. Consequently, a longitudinal area appears as a thickened band over the embryonic disk area, which is known as the **primitive streak**. The proliferation and differentiation of the cells in the primitive streak region result in the formation of the third germ layer – the **mesoderm**, which subsequently spreads laterally to lie between the trophectoderm and the extraembryonic endoderm. The initiation of the mesoderm usually occurs on Day 9 of pregnancy in the pig (Patten, 1948) and immediately precedes the implantation process, which involves attachment between the trophoblast and the uterine epithelium.

Along with the development of the three germ layers, three extraembryonic membranes are formed in the pig (Perry, 1981). The trophectoderm fuses with the avascular outer layer of the extraembryonic mesoderm to form the **chorionic membrane**, which when vascularized supports the growth, nutrition, respiration and excretion of the embryo throughout the gestation. The **amniotic membrane** is also derived from the trophectoderm and the avascular outer layer of the extraembryonic mesoderm, and surrounds the embryo proper. The amniotic fluid can protect the embryo from desiccation and mechanical shock. An outgrowth of embryonic hindgut generates the allantoic membrane that consists of the extraembryonic endoderm and the vascular inner layer of the extraembryonic mesoderm. The allantoic fluid is connected with the urinary bladder of the fetus and is, therefore, the storage place of fetal waste products. As the allantois expands, it fuses with the chorionic membrane to form the **chorioallantoic placenta**.

MATERNAL RECOGNITION OF PREGNANCY

In luteal-dependent mammalian species like the pig, maintenance of the corpora lutea is essential during the whole gestation period. However, in all mammalian species, prolongation of luteal activity of pregnancy beyond the normal time of luteolysis is essential for the establishment of pregnancy and early embryonic development. Maternal recognition of pregnancy is the mechanism by which the developing conceptus signals its presence to the mother, resulting in the prolongation of functional activity of the corpus luteum and, therefore, the maintenance of pregnancy (Perry et al., 1973). Pregnancy recognition occurs before the embryonic tissue becomes intimately attached to the uterine epithelium, and is therefore distinct from implantation. The signal is prolactin in rodents (Cross et al., 1994) and chorionic gonadotropin in primates and horses (Niswender and Nett, 1988; Hearn et al., 1991), and those signals are luteotrophic. In some domestic mammals such as pigs, sheep and cows, the production of luteal progesterone is maintained not by luteotrophic factors, but rather by antiluteolytic factors that prevent the luteolytic effect of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which triggers regression of the corpus luteum in non-pregnant animals. The signal is trophoblast-derived estrogen in the pig, and interferon-tau (IFN-τ) (previously named trophoblast protein-1) in sheep and cows (Bazer et al., 1997 and 1982; Geisert et al., 1994b).

There is little doubt that $PGF_{2\alpha}$, originating from the uterine endometrium, is the luteolytic factor that limits the lifespan of the corpus luteum during the estrous cycle (Bazer et al., 1986 and 1989). Upon receiving the pregnancy recognition signal from the developing embryo(s), an increase in $PGF_{2\alpha}$ synthesis by the endometrium is prevented in

sheep and cows; in contrast, endometrial production of $PGF_{2\alpha}$ in the pig is directed from the venous drainage (endocrine) to the uterine lumen (exocrine) (Figure 1.2; Bazer et al., 1982 and 1994b). However, the complete mechanism by which estrogen directs the secretion of $PGF_{2\alpha}$ into the uterine lumen during pregnancy is unknown. During the period of maternal recognition of pregnancy, porcine blastocysts undergo rapid expansion and initiation of elongation (Perry & Rowlands, 1962; Dhindsa & Dziuk, 1968; Anderson et al., 1978; Geisert et al., 1982b; Stroband and Van der Lende, 1990).

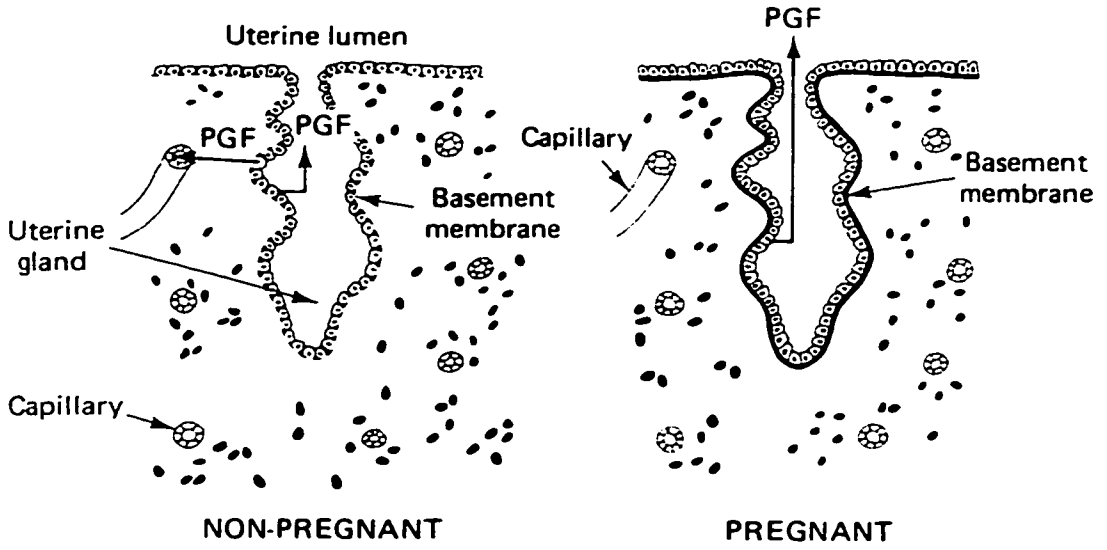


Figure 1.2 Endometrial secretion of $PGF_{2\alpha}$ in non-pregnant (left) and pregnant (right) pigs (Bazer et al., 1982). Upon receiving the pregnant signal from developing embryos, endometrial secretion of $PGF_{2\alpha}$ is directed from the venous drainage (endocrine) to the uterine lumen (exocrine).

BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG

A. Blastocyst Expansion and Elongation

In some mammalian species, blastocysts undergo expansion after they have hatched from the zona pellucida and before they proceed to implantation. The extent to which mammalian blastocysts expand is highly variable and species-dependent. The blastocysts of the human and laboratory rodents show little or no expansion before they implant either interstitially or eccentrically in the uterine endometrium. In contrast, the blastocysts of the rabbit, dog, cat and most livestock species increase their size by several orders of magnitude prior to central implantation (Biggers, 1972). In the extreme, blastocysts of the pig undergo pronounced expansion and subsequent elongation between Day 9 and Day 14 of gestation (Perry & Rowlands, 1962; Anderson, 1978). During this period, the porcine blastocyst transforms from a sphere of approximately 650 μm in diameter to a filamentous form that is up to 100 cm in length.

The capacity to synthesize estrogen is acquired when porcine blastocysts expand to the size of 5-6 mm in diameter, which is temporally associated with the formation of mesoderm (Fischer et al., 1985; Pusateri et al., 1990; Wilson and Ford, 1997). Blastocyst elongation appears to be programmed by endogenous development cues in the embryo, because blastocysts only elongate after they have reached the size of about 10 mm in diameter (Morgan et al., 1987a & 1987b). The 10-mm stage in conceptus development is coincident with the differentiation and expansion of extraembryonic mesoderm (Geisert et al., 1982b; Gupta et al., 1996), which is therefore believed to be the driving force for the cellular remodeling of blastocyst elongation (Geisert and Yelich, 1997). Upon reaching a diameter of about 10 mm on Day 11.5 of pregnancy, porcine

blastocysts undergo a rapid transition from spherical to tubular and filamentous forms (Geisert et al., 1982b), which precedes the attachment to the endometrium. During the initial elongation of pig blastocysts from Day 10 to 12, it appears that cellular remodeling rather than cellular hyperplasia is responsible for morphological changes (Geisert et al., 1982b). The actin cytoskeleton plays an important role in the modification of trophoblast cell shape in elongating porcine embryos (Mattson et al., 1990). Along with the elongation process, the biochemical and physiological activities of blastocysts are increased, which is evident by the onset of estrogen (Gadsby et al., 1980; Fischer et al., 1985) and protein synthesis (Godkin et al., 1982). The onset of embryonic estrogen synthesis is followed by a significant increase in intraluminal calcium content and a rapid release of secretory vesicles from uterine epithelial cells into the lumen (Geisert et al., 1982a; Stroband & Van der Lende, 1990).

B. Blastocyst Attachment/Implantation

Implantation of embryos in the wall of the uterus is the result of a series of complex interactions between the embryo and the uterus, which begins with the apposition of the blastocyst to the uterine epithelium and ends with the formation of a definitive placenta. The detailed process of implantation varies considerably in different species (Weitlauf, 1988), and can be divided into two steps, attachment and penetration. The attachment of embryos to the uterus involves a progressive interdigitation of microvilli between the trophoblast and the uterine epithelium, which occurs as two phases, apposition and adhesion. Adhesion between the two surfaces develops as the apposition phase progresses. Penetration (invasion) of the uterine wall by implanting embryos occurs in many mammals, such as the human, Chimpanzee, and laboratory

rodents (Weitlauf, 1988). However, porcine implanting embryos normally do not penetrate into the uterine wall and only remain attached to the uterine epithelium, as in other domestic species with an epitheliochorial type of placentation (Burghardt et al., 1997; Keys & King, 1990; Dantzer, 1985; Guillomot et al., 1981).

In the pig, intrauterine migration of embryos occurs between Day 7 and 12 of gestation (Dhindsa et al., 1967) (Fig. 1.1). The trophoblast and the uterine epithelium become closely apposed to each other as soon as blastocyst elongation is complete (Flood, 1991). The process of adhesion, beginning around Day 14, initiates in the region of the embryonic disc and progresses toward the trophoblastic tips (Dantzer, 1985; Keys and King, 1990). Based on morphological observations (Dantzer et al., 1981; Keys and King, 1990), the attachment between the blastocyst and the uterus is facilitated by the development of epithelial protrusions in the endometrium enclosed by trophoblastic caps. The formation of such structures is believed to serve to immobilize the elongated blastocysts. Adhesion between maternal and fetal epithelial cells is evident by the microvillous interdigitation between the uterine epithelium and the trophoblast by Day 16 (Dantzer, 1985; Keys and King, 1990). This process of attachment is accomplished over the entire surface of the embryo/conceptus by Day 26 of pregnancy (Amoroso, 1952). Discussion of the further development of the embryo proper is beyond the scope of this review.

POSSIBLE MECHANISMS REGULATING BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG

An essential step during mammalian pregnancy is the implantation of the blastocyst, which is initiated when the trophoblast attaches to the epithelium of the uterus. Before this point, the embryo develops during its migration from the fertilization site in the oviduct to the implantation site in the uterus. Thereafter, the developing embryo becomes dependent on the maternal environment for its continued development to term. Many factors have been implicated in the processes leading to the establishment of pregnancy and implantation in mammals, including hormonal and non-hormonal factors. Hormonal factors include progesterone, luteolytic factors and embryonic signals, which are responsible for maintaining the corpus luteum. Non-hormonal agents include adhesion molecules, vasoactive agents, tissue-remodeling enzymes [matrix metalloproteinases (MMPs)] and tissue inhibitors of MMP (TIMPs), growth factors and cytokines, which are involved in the pre-attachment period and the apposition, adhesion and invasion of the blastocyst.

Uterine secretion (uterotrophe) plays important roles during pregnancy in the pig. It is the major source of embryonic nutrition, since porcine trophoblasts are non-invasive and fail to make direct contact with the maternal blood supply. This type of nutritional exchange is relatively low in efficiency in comparison to the hemotrophe nutrition in species such as the mouse and human, but it is enhanced by the larger contact surface area formed between the trophoblast and the uterine epithelium in the pig. In addition, the formation of a non-invasive placenta in the pig is regulated by the components of uterine secretion together with the embryonic products.

A. Steroids and Their Receptors

One of the most important and earliest factors secreted by the pig embryo is estrogen, which is the signal for maternal recognition of pregnancy. Porcine blastocysts begin producing estrogen on Days 11-12 of gestation (Perry et al., 1976; Ford et al., 1982; Pusateri et al., 1990), a period characterized for the most diverse sizes of littermate conceptuses (Anderson, 1978; Pusateri et al., 1990). The expression of three enzymes involved in estrogen synthesis, $P450_{scc}$, $P450_{17\alpha}$ and $P450_{arom}$, has been detected in the trophectoderm (TE) cells of embryos with a minimum size of ~ 6 mm in diameter (Conley et al., 1992; Wilson and Ford, 1997). It appears that the rate-limiting enzymes in estrogen synthesis are $P450_{17\alpha}$ and $P450_{arom}$ in the pig conceptus, since expression of these two enzyme is closely linked with the estrogen content of the conceptus (Conley et al., 1992 & 1994; Ko et al., 1994; Green et al., 1995, Yelich et al., 1997a). At the time of blastocyst elongation, the receptor for estrogen is up regulated in the uterine epithelium (Geisert et al., 1993), whereas it is not detected in the early conceptus by RT-PCR (Yelich and Geisert, 1997). Therefore, it appears that the embryonic estrogen has an indirect effect on trophoblastic elongation. The brachyury gene, the marker for mesoderm differentiation (Herrman et al., 1990), is expressed coincidentally with the expression of $P450_{17\alpha}$ and $P450_{arom}$ (Yelich et al., 1997a), but its relationship with estrogen synthesis and blastocyst elongation is not yet clear.

During early pregnancy in the pig, morphologically advanced blastocysts appear to have a greater survival potential than their less developed littermates (Pope et al., 1982; Pope & First, 1985). The importance of synchrony between blastocyst development and uterine environment has been demonstrated through the application of

embryo transfer techniques in the pig (Geisert et al., 1991c). It is generally believed that estrogen derived from more advanced conceptuses changes the uterine milieu at the time of blastocyst elongation, which could be detrimental to less developed littermates (Dziuk, 1987; Pope et al., 1990; Pope, 1994). Indeed, embryonic estrogen has a modulatory role on the expression of some components of uterine histotrophe, such as retinol-binding protein (RBP) and uterine serpin (Trout et al., 1992) but not uteroferrin (Simmen et al., 1991). However, most of the major histotrophe components appear to be synthesized by the endometrium in response to progesterone (Roberts et al., 1993).

Differential production of embryonic estrogen has been proposed to be one of the factors that cause the Chinese Meishan breed to be more prolific than the American Yorkshire breed (Wilson and Ford, 1997). Chinese Meishan embryos are smaller and contain fewer trophectoderm cells than American Yorkshire embryos on Day 12 of gestation (Anderson et al., 1993; Rivera et al., 1996). Since estrogen synthesis occurs exclusively in the TE (Conley et al., 1994) and at a similar level per cell (Anderson et al., 1993), Meishan embryos produce much less estrogen than Yorkshire embryos on Days 11-12. Therefore, the uterine histotroph composition would be less dramatically changed in Meishans, allowing more embryos to survive (Wilson and Ford, 1997). This theory is also supported by evidence that there is less IGF-I in the uterine luminal fluid of the Meishan as compared to the Yorkshire and IGF-I might stimulate $P450_{arom}$ activity (Ko et al., 1994; Hofig et al., 1991a).

Expression of estrogen receptor (ER) mRNA in the endometrium of pregnant gilts was greatest on Day 10, followed by a decline on Day 15, and another increase on Day 18, but stayed low during the remainder of pregnancy (Geisert et al., 1993).

However, endometrial expression of progesterone receptor (PR) was highest on Days 0-5, decreased on Day 10, and reached a nadir on Day 12 that was maintained throughout the remainder of the estrous cycle (Geisert et al., 1994a). This dynamic change of PR and ER in the endometrium is consistent with the physiological role of embryonic estrogen in the maternal recognition of porcine pregnancy.

During blastocyst elongation, porcine embryos also produce PGF2α (Lewis and Waterman, 1983), although expression of the receptors for progesterone, estrogen, oxytocin and PGF2α have not been detected in embryonic tissues (Yelich et al., 1997b).

B. Role of Retinol and Retinoic Acid in Embryonic Morphogenesis

Retinol-binding protein (RBP) is present within the uterine lumen (Clawitter et al., 1990; Roberts and Bazer, 1988), and embryos (Trout et al., 1991; Harney et al., 1993) during rapid blastocyst elongation in the pig. Embryonic estrogen derived from more advanced embryos could trigger a massive increase in uterine expression and secretion of retinol-binding protein (Roberts et al., 1993; Trout et al., 1992). As a result, there would be an abrupt increase of retinol in the uterine lumen at a time when less developed embryos are least able to contend with excessive exposure to retinol, a potential teratogen (Lammer et al., 1985) and embryotoxic agent (Thompson et al., 1993).

RBP is believed to act as an acceptor and delivery vehicle of retinol from the plasma to its target tissues (Blomhoff et al., 1990), and also functions in the transport and regulation of cellular retinoids (Harney et al., 1994a & 1994b). One of the cytoplasmic metabolites, retinoic acid (RA) is known as an endogenous morphogenic substance (Ross, 1991). RA binds to a group of transcription factors called RA receptors (RARs),

which belong to the steroid/thyroid receptor family (De Luca, 1991) and consist of three subclasses, RARα, RARβ and RARγ (Mattei et al., 1988a and 1988b; Ishikawa et al., 1990). RARs specifically bind to RA response elements in the vicinity of target genes to activate gene expression (Linney, 1992). RA has been demonstrated to regulate the expression of tissue transglutaminase (Chiocca et al., 1989), extracellular matrix components (De Luca, 1991; Ross et al., 1994), proteases and their inhibitors (Adler et al., 1990; Tienari et al., 1991), adhesion molecules (Agura et al., 1992), and growth factors and their receptors (Jetten, 1980; Sporn et al., 1986). RA stimulates the expression of transforming growth factor β (TGFβ), which in turn acts as a chemoattractant to modify extracellular matrix components (Kimelman & Kirscher, 1987; Roberts and Sporn, 1988). Recently, Yelich et al. (1997) demonstrated that RBP, RARs and TGFβ3 are expressed temporally in elongating blastocysts during the peri-implantation period, suggesting their involvement in morphogenesis of porcine embryos. It is not surprising that RA has been implicated as one of the most promising candidates for embryonic tissue remodeling during blastocyst elongation (Roberts et al., 1993; Geisert and Yelich, 1997).

C. Trophoblast-Uterine Epithelium Adhesion

At implantation, the previously non-adhesive apical surface of the trophectoderm becomes adhesive. Increased knowledge of cell adhesion mechanisms, which occur during lymphocyte extravasation (Lasky, 1995), has led to extensive investigation of the molecular mechanism(s) underlying the adhesion process in the mouse and human. The present theory is that carbohydrate-lectin interactions might mediate initial blastocyst adhesion, which is then stabilized by binding of integrins to

their extracellular matrix (ECM) ligands (Aplin, 1997; Cross et al., 1994). The local loss of anti-adhesion molecules such as MUC-1 on the epithelial surface may contribute to the establishment of the receptive uterine state, and therefore facilitates attachment between the trophoblast and the uterine epithelium (Surveyor et al., 1995). Adhesion molecules implicated in the attachment process include integrins, ECM components, cadherins, lectins, heparin sulfate proteoglycans (Aplin, 1997; Lessey et al., 1996; Wegner and Carson, 1994; Cross et al., 1994; Damsky et al., 1993).

Integrins are a large family of cell surface glycoproteins involved in both cell-cell and cell-ECM interactions, and function as transmembrane heterodimers consisting of α and β subunits that are associated with cytoskeletal and cell signaling proteins (Gille and Swerlick, 1996; Hynes, 1992). At least 24 integrin heterodimers could be formed with the known 16 α subunits and 9 β subunits, and 9 of them have been identified in human uterine epithelium (Table 1.1; Aplin, 1997). Integrins are modulated during the reproductive cycle and early pregnancy in humans and rodents (Lessey et al., 1992; Tabibzadeh, 1992; Nishida et al., 1991), and peak expression of $\alpha v\beta 3$ and $\alpha 4\beta 1$ integrins in human uterine epithelium is coincident with the implantation window (Lessey et al., 1994). In the pig, some integrin subunits and ligands were detected in the uterine epithelium and trophoblast of estrous and early pregnant gilts (Table 1.2; Burghardt et al., 1997; Bowen et al., 1996 & 1997). Based on the spatial and temporal expression of integrin subunits and ligands at the implantation sites in the pig, integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 1$, members of fibronectin receptors, have been implicated as the possible

Table 1.1 Integrins in human uterine epithelium and trophoblast, and their ligands

	Uterine epithelium	Trophoblast#	Ligands@
α1β1	+r*	e	LN,COL,PE
α2β1	+		COL
α3β1	+	e, v	FN,COL,LN
α4β1	+r		FN,VCAM
α5β1		e	FN
α6β1	?	?	LN
α9β1	+		TN
α6β4	+	v	LN
αvβ1	?	?	FN,VN
αvβ3	+r	e	FN,VN,OS,Vwf,FIB,BSP1,PE,PECAM-1
αvβ5	+		FN,VN,OS
αvβ6	+r		FN,TN

*r, regulated expression; #e and v, extravillous and villous expression, respectively; @BSP1, bone sialoprotein 1; COL, collagens; FIB, fibrinogen; FN, fibronectin; LN, laminins; OS, osteopontin; PE, perlecan (heparan sulfate proteoglycan); PECAM-1, platelet endothelial cell adhesion molecule; TN, tenascin C; VCAM, vascular cell adhesion molecule; VN, vitronectin; vWF, von Willebrand factor. '?' refers to integrins for which the constituent subunits are present but the specific association not demonstrated.

candidates for trophoblast-uterine epithelium attachment (Burghardt et al., 1997). In addition, expression of porcine MUC-1 in the uterine epithelium is down-regulated during the peri-implantation period as in humans and rodents (Burghardt et al., 1997; Aplin, 1997). Further studies are needed to determine which integrins are expressed specifically on the apical surface of porcine uterine epithelium.

It is now known that integrins serve as receptors for extracellular matrix (ECM) proteins and also transduce signals into the cell through focal adhesion kinase (FAK) (Yurochko, 1997; Juliano, 1996, Brown and Hogg, 1996). Integrin signaling events regulate cellular processes such as proliferation, apoptosis and motility (Cary and Guan, 1999). New evidence suggests that transmembrane-4 superfamily (TM4SF) proteins may facilitate integrin-signaling transduction by acting as linkers between extracellular integrin α subunit domains and intracellular signaling molecules (Hemler, 1998).

Table 1.2 Subunits of integrins and their ligands in porcine uterine epithelium and trophoblast#

	Subunits of integrins			Ligands
	H + C	H + M	L	
Uterine epithelium	αv; β3	α4; α5; β1	α1; α3	Vitronectin
Trophoblast	α1; α4; α5; αv; β1; β3			Vitronectin; Fibronectin
Sites of attachment	α4; α5; αv; β1; β3			Vitronectin; Fibronectin

#H+C, high and constitutive expression; H+M, high and modulated expression with maximal expression during maternal recognition of pregnancy (Days 10 to 15); L, low or no expression.

D. Formation of Porcine Non-invasive Placenta

Trophoblast cells are invasive in human and rodents, and produce two types of proteolytic enzymes, matrix metalloproteinases (MMPs) and a urokinase-type plasminogen activator (uPA), that degrade the ECM during endometrial invasion (Cross et al., 1994). At least 18 members have been characterized in the family of MMPs, which play important roles in wound healing, bone resorption, mammary involution, tumor invasion and matastasis, and pregnancy and parturition (Table 1.3; Parsons et al., 1997; Pei, 1999; Ryu et al., 1999; Pendas et al., 1997; Sato et al., 1997; Puente et al., 1996; Stolow et al., 1996). MMP-9 production and activation by both human and mouse trophoblasts peak during maximal invasive behavior *in vivo*, and are also required for trophoblast invasiveness *in vitro* (Harvey et al., 1995b; Shimonovitz et al., 1994; Behrendtsen et al., 1992; Librach et al., 1991). The uPA can activate the latent forms of matrix metalloproteinases (MMPs) through the PA-plasmin-MMPs cascade, but it does not appear to be essential for implantation (Carmeliet et al., 1994). MMP-9 has emerged as the main proteinase involved in implantation of human and mouse embryos (Alexander et al., 1996). Implantation in the pig is non-invasive and characterized by interdigitation between the trophoblast and the uterine epithelium, but porcine trophoblast becomes invasive when embryos are transferred to ectopic sites (Samuel, 1971 & 1972; Samuel and Perry, 1972). Furthermore, porcine embryos secrete uPA and MMP-2 during the peri-implantation period (Mullins et al., 1980; Fazleabas et al., 1983; Chamberlin and Menino, 1995), and very weak expression of uPA and MMP-9 transcripts is detected in the trophoblast (Menino et al., 1997). Trophoblast cells derived from porcine embryos of

Table 1.3 Matrix metalloproteinases and their substrates

MMP	Other name	Main substrate(s)
MMP-1	Collagenase-1	Fibrillar collagens
MMP-2	Gelatinase A (72 kDa)	Type IV and V collagens, fibronectin
MMP-3	Stromelysin 1	Laminin, fibronectin, non-fibrillar collagen
MMP-7	Matrilysin	Laminin, fibronectin, non-fibrillar collagen
MMP-8	Collagenase-2	Fibrillar collagens
MM-9	Gelatinase B (92 kDa)	Type IV and V collagens
MMP-10	Stromelysin 2	Laminin, fibronectin, non-fibrillar collagen
MMP-11	Stromelysin 3	Serpin (protease inhibitor)
MMP-12	Metalloelastase	Elastin
MMP-13	Collagenase-3	Fibrillar collagens
MMP-14	Mt1-MMP	ProMMP-2
MMP-15	Mt2-MMP	ProMMP-2
MMP-16	Mt3-MMP	ProMMP-2
MMP-17	Mt4-MMP	
MMP-18	Collagenase	Interstitial collagens
MMP-19		Gelatin
MMP-20	Enamelysin	Amelogenin
Mt5-MMP		MMP-2

Day 14 produce MMP-9 and –2 *in vitro* (Chai and Dixon, unpublished results). Therefore, the degradation of ECM components must be tightly controlled in order to support the formation of the noninvasive, epitheliochorial placenta in the pig, which could be accomplished by the endometrial secretion of a variety of protease inhibitors (Fig. 1.3).

Tissue inhibitors of MMPs (TIMPs), including TIMP-1, -2, -3 and –4, are the specific inhibitors for the MMPs (Fortunato et al., 1998; Leco et al., 1997). In the mouse, TIMP-3 transcripts were predominantly expressed in the decidua immediately adjacent to the embryo, and the expression of TIMP-1 and –2 was not detected at the sites of implantation (Harvey et al., 1995b; Reponen et al., 1995). Therefore, it appears that TIMP-3 is the principal endometrial inhibitor that regulates the extent of embryonic penetration in mice (Alexander et al., 1996; Leco et al., 1996). Porcine embryos and uteri also express TIMP-1, -2 and –3 during the period of blastocyst attachment. TIMP expression is abundant in porcine embryos, especially in the trophoblast, and is also localized in the stroma but not in the epithelium of the uterus (Menino et al, 1997). TGFβ is a potent inducer of TIMP-1 and –2 (Graham et al., 1994) and has been proposed as a negative regulator of trophoblast invasion (Cross et al., 1994). Both TGFβs and their receptors are produced in porcine embryos during the attachment period (see section on growth factors in this review). It is possible that embryonic production of TFGβ may participate in inducing TIMP expression in the uterus and/or strengthening the TIMP production in the trophoblast. Therefore, the formation of non-invasive placentae in the

pig could be mainly controlled from the embryos themselves by strong expression of TIMPs and TGFβ and weak expression of MMPs and uPA by the trophoblast.

The presence of inhibition of both uPA and plasmin in porcine uterine fluid could also nullify the potential extracellular proteolysis by uPA and plasmin (Mullins et al., 1980; Fazleabas et al., 1983). During blastocyst attachment in the pig, an inhibitor containing a Kunitz domain is secreted, which is specific for at least three serine proteases (plasmin, trypsin and chymotrypsin) (Duffy et al., 1997). Another group of plasma serine protease inhibitors, the inter-α-trypsin inhibitor (IαI) family, has also been detected in the endometrium during the elongation and attachment (Geisert et al., 1996; Diederich et al., 1997). The IαI family functions as complexes consisting of two types of subunits, heavy chain (H) and light chain. The light chain is also known as bikunin because it contains two Kunitz domains on its amino acid sequence. So far, three members of the IαI family has been identified, which include IαIH1- IαIH2-bikunin, IαIH2-bikunin and IαIH3-bikunin. The subunits in those inhibitors are connected to each other by inter-chain glycosaminoglycan bonds. In addition to binding to hyaluronic acid (HA), all IαI heavy chains contain a von Willebrand type-A domain that functions as a target for adhesion molecules such as integrins, collagen, proteoglycans and heparin. It is obvious that the IαI family functions not only as protease inhibitors but also as stabilizers for the ECM (Geisert and Yelich, 1997).

Two other protease inhibitors secreted by the porcine endometrium are antileukoprotinease (ALP) for a uterine elastase/cathepsin G protease (Simmen et al., 1992) and uteroferrin-associated proteins (UfAPs) that are related to the serpin family of

protease inhibitors (Murray et al., 1989; Malathy et al., 1990). The porcine endometrium also secretes many other enzymes including lysozyme, leucine-aminopeptidase, β-hexosaminidase, and cathepsins B1, D, E and L, which have anti-bacterial function and degrade proteins for embryo uptake (Geisert and Yelich, 1997). The cathepsins are lysozymal cysteine proteases that have been implicated as modulators of invasive implantation of the rat and cat (Elangovan and Moulton, 1980; Li et al., 1992). Cathepsin L, expressed in the porcine uterus, has a high affinity for collagen and elastin, which suggests that it may play a role in attachment between the trophoblast and the epithelium.

E. Regulators of Implantation: Growth Factors and Their Receptors

Growth factors are known to play important roles in the control of the processes of cellular proliferation, differentiation and morphogenesis during pre-implantation mammalian embryonic development, which has been extensively reviewed (Kaye PL, 1997; Kaye and Harvey, 1995; Schultz and Heyner, 1993). These growth factors are believed to function in an autocrine and/or paracrine fashion within the embryo itself or through interactions with the uterus. This review will not attempt to provide an exhaustive catalogue of these factors, but will focus on growth factor systems that are, or are likely to be, important during implantation in mammals, particularly in the pig. Recently, some of these growth factors have been demonstrated to be relevant to the process of implantation in mammals (Nie et al., 1997, Polan et al., 1995; Harvey et al., 1995a). Geisert and Yelich (1997) have reviewed the presence of growth factors and their receptors during the peri-implantation period in the pig, although little or no information exists at this point regarding their functions.

1. Insulin-like growth factor family

This family of growth factors has been extensively studied. Insulin-like growth factor-I (IGF-I) and IGF-II have a high degree of amino acid sequence homology with proinsulin, and are transported in extracellular fluids by binding to a group of IGF-binding proteins (IGFBPs). IGF-I receptor (IGF-IR) and IGF-IIR are structurally unrelated to each other, and IGF-IIR also carries a separate binding site for mannose-6 phosphate (M6P) (Kay, 1997).

Temporal expression patterns of IGF-I, IGF-II, IGFBP-2 and IGF-IR have been demonstrated in the endometrium of cyclic and pregnant pigs, and constitutive levels of IGF-II and IGF-IR are observed (Letcher et all, 1989; Simmen et al., 1992) (Table 1.4). In contrast, IGF-I and -II have also been detected in embryos, although little or no expression of IGFBP-2 is detected in embryos or placenta (Letcher et all, 1989; Simmen et al., 1992; Green et al., 1995). Expression of IGF-IR mRNA was demonstrated in peri-implantation embryos using a combination of RT-PCR and Southern hybridization

Table 1.4 Expression of IGF-I, IGF-II, IGF-IR, IGF-IIR and IGFBP-2 in the endometrium and the embryo of pigs.

	IGF-I	IGF-II	IGF-IR	IGF-IIR	IGFBP-2
Endometrium	+	+	+	-	+
Embryo	+	+	+	+	-

methods (Green et al., 1995), although it was not detected using less sensitive immunohistochemistry and autoradiography methods (Chastant et al., 1994). However, the expression of IGF-IIR was only observed in the trophoblast but not in the endometrium (Chastant et al., 1994). These results indicate that the IGF system modulates the interactions between the endometrium and embryos in a paracrine and/or autocrine fashion (Figure 1.3).

Endometrial expression of IGF-I mRNA and IGF-1 content in the porcine uterine lumen peak at Day 12 of pregnancy, which is concomitant with maximal estrogen production by embryos (Ko et al., 1994; Simmen et al., 1992; Letcher et al, 1989; Tavakkol et al., 1988). Moreover, $P450_{arom}$ levels in porcine embryos are highly correlated with the ratio of IGF-I to IGF-II in the uterine lumen (Ko et al., 1994). Furthermore, it has been demonstrated that induction of the functional activity of $P450_{arom}$ in porcine embryos by IGF-I is dependent on the development stage of these embryos *in vitro* (Hofig et al., 1991b). Therefore, endometrial production of IGF-1 might play a potential role in modulation of porcine embryonic steriodogenesis during the peri-implantation period.

2. Epidermal growth factor family

EGF is a small polypeptide of 53 amino acids, and stimulates the proliferation of epidermal and epithelial cells in whole animals and of a variety of cell types *in vitro*. Many polypeptides structurally and functionally related to the EGF have been described (Lim et al., 1997; Das et al., 1995), which include transforming growth factor-α (TFG-α), heparin binding-EGF (HB-EGF), amphiregulin (Ar), beta-cellulin, epiregulin, and

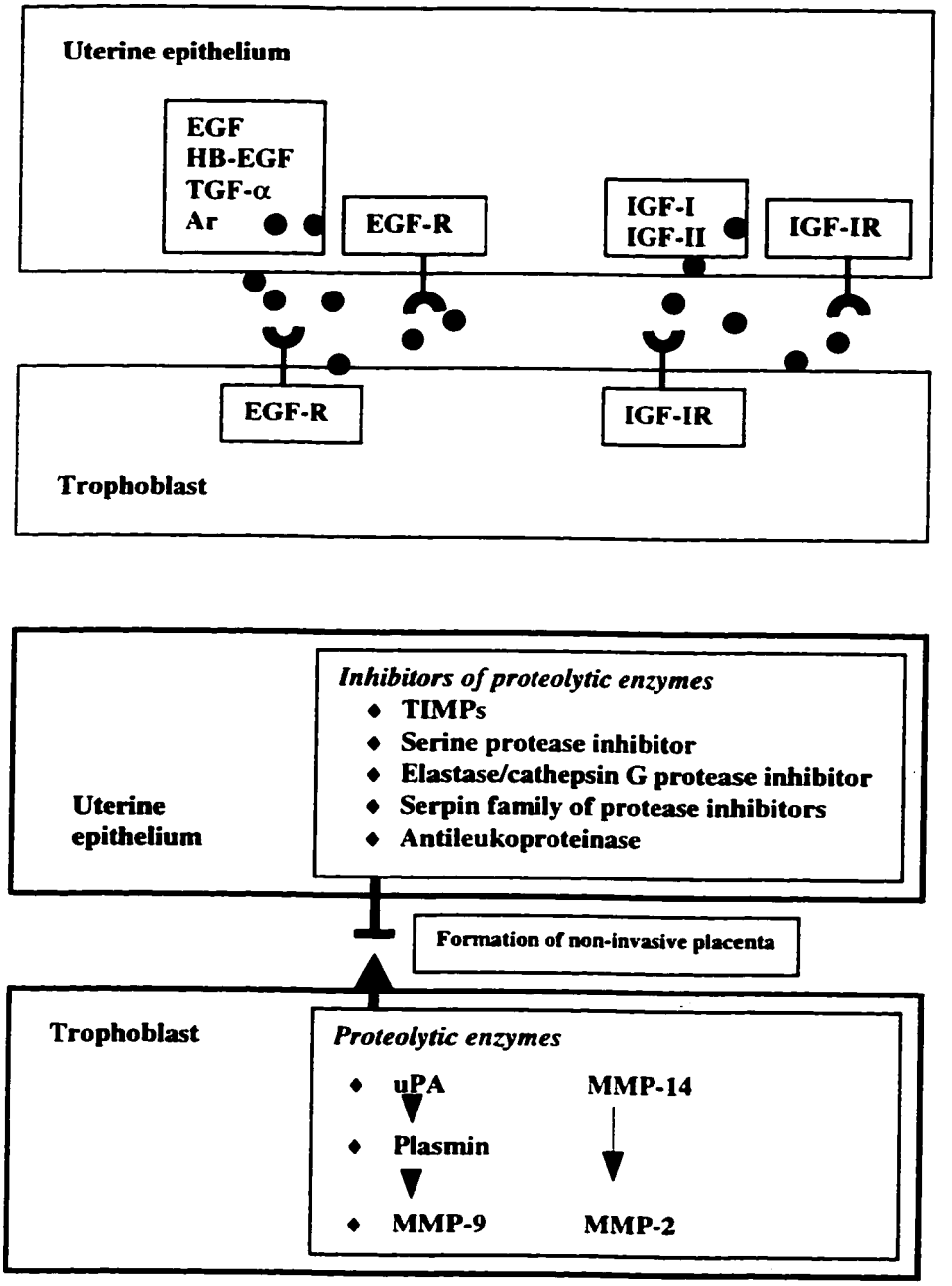


Figure 1.3 Upper panel, autocrine and paracrine actions of growth factors (EGF family and IGF-I family) at the interface between the trophoblast and the uterine epithelium during early pregnancy in the pig. Lower panel, production of proteolytic enzymes and their inhibitors during the attachment of the two surfaces, which might contribute to the formation of the non-invasive placenta in the pig.

heregulins. Four receptors for the EGF family are identified, which include erbB1 (EGF receptor), erbB2, erbB3, and erbB4. TGF-α and EGF have an equal affinity for the erbB1 (Lim et al., 1997). This family of growth factors have been implicated to be important for implantation in many species such as the human, mouse, rat, cow, sheep and cat (Yoo et al., 1997; Boomsma et al., 1997; Das et al., 1997; Johnson et al., 1996; Chia et al., 1995; Gharib-Hamrouche et al., 1995).

Expression of erbB1, EGF, TFG-α, and Ar at mRNA level, but not that of HB-EGF, has been detected in the oviduct and endometrium of cyclic and pregnant pigs (Kennedy et al., 1994) (Table 1.5). The erbB1 mRNA was detected at all stages of embryo development examined (Vaughan et al, 1992), but EGF expression started at Day 15 and continued to increase up to Day 22. Interestingly, TGF-α was expressed only at Day 8, 10 and 12, with a peak at Day 12, which is coincident with the maternal recognition of pregnancy in the pig. Recently, HB-EGF-like growth factors have been isolated from porcine uterine luminal flushings, which are expressed in endometrial tissues of pregnant and cycling pigs (Kim et al., 1995). In addition, high molecular mass forms (25 kDa) of epidermal growth factor have been detected in pig uterine secretions (Brigstock et al., 1996). These data indicate that this system of growth factor functions in an autocrine and paracrine manner during porcine blastocyst elongation and attachment (Fig. 1.3).

3. Transforming growth factor β

TGFβ is one member of a superfamily including activin and inhibin (Pfeilshifter, 1990). Three forms of TGFβ are identified, TGFβ-1, TGFβ-2 and TGFβ-3,

and two types of TGFβ receptors, TGFβ-IR and TGFβ-IIR, are found in most cells in different ratios. They mediate many key events in normal growth and development. In addition to its effect on cell proliferation and differentiation, TGFβ increases matrix synthesis and decreases matrix degradation by regulating the production of MMPs, TIMPs, tissue PA and PA inhibitor (Pfeilschifter, 1990). TGFβ is involved in mesoderm induction and body patterning in *Xenopus* and *Drosophila* embryos (Hoffman, 1991; Kimelman et al., 1992). Expression of TGFβ has also been demonstrated at implantation sites in the human, mouse and sheep (Graham et al., 1992; Dore, 1995; Stager et al., 1991), but little is known about their effects on mammalian embryo development.

Table 1.5 Expression of the EGF family in the oviduct, the endometrium and the embryo of pigs.

	Oviduct	Endometrium	Embryos							
			D7	D8	D10	D12	D15	D17	D18	D22
ErbB1	+	+	+	+	++	++	++	++	++	++
EGF	+	+	-	-	-	-	+	++	+++	++++
TFG-α	+	+	-	+	++	+	-	-	-	-
HB-EGF	-	-								
Ar	+	+								

TGFβ and its receptors have been shown to be differentially expressed during the peri-implantation period in the pig. Both TGFβ2 and TGFβ3 are expressed in the trophoblast from Days 10-14 of gestation when blastocysts undergo rapid elongation (Gupta et al., 1996). During this period, TGFβ-2 expression is dramatically decreased, whereas TGFβ-3 expression is constant. In porcine embryos, expression of TGFβ-3 is demonstrated to be developmentally regulated, and it increases gradually from the 2-mm spherical to 8-mm spherical stage and remains constant during the remainder of blastocyst elongation (Yelich et al., 1997b). In addition, TGFβ-IR is consistently expressed, although TGFβ-IIR expression is abruptly decreased in porcine embryos during the peri-implantation period (Gupta et al., 1996). TGFβs could be involved in initiation or progression of mesoderm migration in porcine conceptuses or in suppression of maternal immune reactions (Gupta et al., 1996).

4. Interferon family

Interferons (IFNs) are multifunctional cytokines, initially defined by their ability to induce a state of resistance against viral infection in target cells (Hauser, 1990). In mammals, they fall into two groups, type I and type II. Type I IFNs consists of a heterogeneous group of molecules sharing sequence homology, and they are encoded by a set of clustered, intronless genes and are expressed in a broad range of cell types. Until now, four subgroups of type I IFNs have been identified, which are IFNα, IFNβ, IFNω and IFNτ. IFNτ (previously called ovine trophoblastic protein) is the first member of the type I IFN family whose primary function is not antiviral and whose expression is physiologically controlled (Bazer et al., 1996). Type II IFN includes only IFNγ, and is

encoded by a single gene with introns. There are three types of receptors identified, IFNαB receptor, IFNα/β receptor (IFNα/β-R) and IFNγ receptor (IFNγ-R).

In the pig, all types of IFNs have been identified except of IFNτ (La Bonnardiere et al., 1994). IFN activity was first demonstrated to be expressed in embryos during Days 11 and 17 of pregnancy, and was also found in uterine flushings during the same period (Cross and Roberts, 1989). Upon further analysis, the major species responsible for the activity was shown to be IFNγ (Lefevre F. et al., 1990). Subsequently, a novel type I IFN gene was also isolated and characterized from a Day 15 pig embryo cDNA library (Lefevre and Boulay, 1993). The protein encoded by this novel gene is shorter and richer in Cys residues than other members of type I IFN family, and is distantly related to all of them. This gene is intronless in the pig genome, and, therefore, it represents the first member of a new subgroup of the type I family. It is unique that porcine trophoblast could be developmentally induced to produce both type I and type II IFNs. However, the precise roles of trophoblastic IFNs remains unknown, although a possible effect on implantation could be predicted by the fact that INFγ is known to up-regulate adhesion molecules in other cells (La Bonnardiere et al., 1994).

5. Macrophage colony-stimulatory factor

Macrophage colony-stimulatory factor (M-CSF or CSF-1) is a glycosylated protein with a molecular weight of 45 kDa (Das and Stanley, 1982) to 90 kDa (Wong et al., 1987) depending on the degree of glycosylation, and stimulates the survival, proliferation and differentiation of monocytes (Stanley et al., 1983). CSF-1 receptor (CSF-1R) is identical to the *c-fms* proto-oncogene product (Sherr et al., 1985), and is a

transmembrane protein with a molecular weight of 165 kDa. A role of CSF-1 in implantation was suggested by observations made in the CSF-1 deficient and osteopetrotic (csf^{op}/csf^{op}) mouse (Cohen et al., 1997; Pollard et al., 1991), which showed a lower implantation rate than wild type mice. These results indicate that the presence of CSF-1 is not essential for successful implantation in mice, which could be due to the functional redundancy of cytokines during the implantation period (Cohen et al., 1997).

In human, CSF-1 is expressed in the endometrium and blastocyst during the peri-implantation period (King et al., 1995; Kauma et al., 1991 and Arceci et al., 1989). The major source of CSF-1 was found to be decidual cells (Jokhi et al., 1995). CSF-1R has been predominantly localized to cytotrophoblastic cells (Jokhi et al., 1993), which suggests that CSF-1 is linked to trophoblast differentiation and proliferation (Hamilton, 1997). Furthermore, *in vitro* studies (Sapi et al., 1996) indicated that expression of CSF-1R could facilitate cellular invasion in mammary epithelial cells.

In the porcine endometrium, CSF-1 mRNA has been shown to increase gradually from Day 15 to term, with the highest level at term. Placental expression of CSF-1 also increased during the gestation stages examined with a peak between Days 20 and 30 (Tuo et al., 1995).

6. Leukemia inhibitory factor

LIF is a multi-functional cytokine and has been shown to regulate the proliferation and differentiation of many types of cells (Stewart, 1994; Shellard et al., 1996). In contrast, it promotes the proliferation but prevents the differentiation of embryonic stem cells *in vitro* (Smith et al., 1988). LIF is a member of a growing family

of cytokines, which includes IL-6, IL-11, Ciliary Neurotrophic factor (CNTF), Oncostatin M (OSM) and Cardiotrophin-1 (CT-1) (Shellard et al., 1996). Their transmembrane receptors are heterodimers and consist of LIF receptor chain and gp 130, which have low and high affinity with these ligands, respectively. The gp130 is responsible for signal transduction of all these family members (Kishimoto et al., 1992).

In mice, LIF expression in the endometrium is up-regulated at the time of blastocyst implantation (Bhatt et al., 1991). Furthermore, LIF null mice have been shown to ovulate normally, but their blastocysts fail to implant. However, their blastocysts are still viable, and could implant and develop to term if they are transferred to wild-type pseudopregnant recipients (Stewart et al., 1992; Shellard et al., 1996). In addition, *in vitro* studies have shown that LIF could down-regulate the production of uPA and MMP activities by mouse embryos from the peri-implantation period (Harvey et al., 1995a). Those results indicate that endometrial expression of LIF is required for the successful implantation. In the absence of LIF, neither embryo attachment nor decidualization of the uterus will occur (Stewart and Cullinan, 1997).

Peak LIF activities were observed in porcine uterine fluids on Day 12 of gestation as well as Days 7 to 13 of the estrous cycle, and IL-6 activity was detected throughout the pre-implantation period and the estrous cycle (Anegon et al., 1994). LIF mRNA was detected in the endometrium at Day 11 but not at Day 6, and it was not detected in the trophectoderm at Day 11. A porcine IL-6 cDNA was cloned, and IL-6 mRNA was first detected in porcine embryos at Days 13 and 21 of pregnancy (Mathialagan et al., 1992). Furthermore, higher levels of IL-6 mRNA were demonstrated in the endometrium than in embryos (Anegon et al., 1994). These results indicate that the

production of LIF and IL-6 is not dependent on the presence of embryos and is mainly derived from the endometrium. Further experiments are needed to clarify the temporal and spatial expression of LIF and IL-6 in the endometrium and embryos of pigs.

7. Interleukin-1 family

Interleukin-1 (IL-1) is a family composed of three structurally related polypeptides, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) (Simon et al., 1995 & 1997). IL-1α and IL-1β recognize the same receptor on target cells and produce similar effects (Dower et al., 1986), although, surprisingly, they share only 26% amino acid homology (Veerapandian, 1992). There are two types of receptors for IL-1, type I (IL-1RI) and type II (IL-1RII), and they function independently at the level of ligand binding because they do not form a heterodimer (Slack et al., 1993).

In both mouse and human endometrium, IL-1α and IL-1β have been localized at the mRNA and protein levels to endometrial macrophages and endothelial cells (Takacs et al., 1988; Simon, et al., 1993). Similar dynamics of IL-1 production were observed in the endometria of pregnant and pseudopregnant mice (Choudhuri and Wood, 1993; De et al., 1993), which suggests that the endometrial IL-1 is hormonally regulated. The IL-1 system has also been shown to present in human and mouse embryos (Kruessel et al., 1997; De los Santos et al., 1996). However, the embryo releases the IL-1 only when it is co-cultured with human endometrial cell-conditioned media, which indicates an obligate role of the endometrium in the regulation of the embryonic IL-1 system (Simon et al., 1997).

Earlier in the development at the human implantation site, IL-1RI was located in the syncytiotrophoblast (ST) and maternal decidua, whereas IL-1β was present in the ST, villous and columnar cytotrophoblast and maternal decidua. IL-1ra was also observed in the intervillous space and maternal decidua (Hu et al., 1992; Simon et al., 1994a). These results suggest that the IL-1 system acts in an autocrine/paracrine manner in human implantation. In addition, *in vitro* studies demonstrated that IL-1 had a stimulatory effect on hCG release from human first-trimester trophoblast cells (Yagel et al., 1989). Therefore, the IL-1 system may exert autocrine/paracrine regulatory effects on hCG secretion by the stimulation of IL-1RI located on ST.

From the studies above, it seems that under the proper progesterone-primed environment, endometrial IL-1RI is synthesized and in turn triggers the production of IL-1 in the trophoblast of the implanting embryo. The interaction between embryonic IL-1 and endometrial IL-1R somehow initiates a series of events, which allows the embryo to attach and invade through the uterine epithelium and basement membrane (Polan et al., 1995). Such implantation events in mice are blocked by the introduction of IL-1ra into the uterine cavity (Simon et al., 1994b), which supports the hypothesis that appropriate interactions between the IL-1 and its receptor at the endometrium and embryo interface are a requisite for successful implantation. However, conflicting results were obtained from studies on mice lacking functional IL-1RI, which did not show any profound alterations in their reproduction (Abbondanzo et al., 1996). Furthermore, the effect of IL-1ra on implantation was not repeated in either wild type or IL-1RI deficient mice.

Recently, IL-1 has been shown to augment the production of LIF in mouse decidual cells (Sawai et al., 1997), IL-6 in human endometrial epithelial cells (Laird et

al., 1994) and CSF-1 in placenta cells (Kauma, 1993). All of these growth factors are also implicated to play a role during implantation, which indicates that a network of growth factors and cytokines might exist in the endometrium to influence embryo implantation.

In the pig, expression of IL-1β has been examined at the mRNA and protein level in embryos recovered from Day 11 to Day 12 of pregnancy (Tuo et al., 1996). The production of IL-1β was observed in porcine peri-implantation embryos of Days 11 to 13 of pregnancy, but not in conceptuses recovered after Day 15 of pregnancy. Further characterization of the IL-1 family in conceptus and endometrium is needed to understand the potential role of IL-1 for the establishment of pregnancy and/or implantation in the pig.

F. Other Factors Secreted in the Uterus during the Peri-Implantation Period

1. Uteroferrin

Uteroferrin is one of the earliest uterine proteins identified within the porcine uterus (Roberts et al., 1986; Roberts and Bazer, 1988). In addition to playing a role in iron transport to the fetus throughout pregnancy, uteroferrin may also serve as a hemotopoietic stem cell growth factor during early embryo development (Bazer et al., 1991; Geisert and Yelich, 1997).

2. Kallikrein, kininogen and kinin system

The activity of kallikrein, one type of kininogenase, is found to increase during the elongation period, which indicates that the kallikrein-kininogen-kinin system might play a role in the trophoblast attachment to the uterine epithelium (Geisert and Yelich,

1997). Kallikreins are serine proteases, and their substrates are kininogens (Bhoola et al., 1992). Kinins are the cleavage products of kininogens, and include bradykinin, kallidin (Lys-bradykinin) and Met-Lys-bradykinin. Kinins are vasoactive peptides that influence many biological processes. They are hypotensive, increase vascular permeability, contract smooth muscle of the intestine and uterus, and increase sperm motility.

In the pig, embryonic estrogen stimulates increases in uterine blood flow, vasodilatation of the capillaries surrounding elongated blastocysts, contraction of the myometrium, and endometrial release of calcium, protein and histamine (Geisert et al., 1990). These processes could be mediated by the kallikrein-kininogen-kinin system in the pig uterus (Geisert and Yelich, 1997).

POSSIBLE SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN MAMMALIAN IMPLANTATION

A. Signal Transduction via Intracellular Receptors

Steroids (progesterone, androgen, and estrogen) can directly diffuse across the plasma membranes of target cells, and their biological effects are mediated by intracellular ligand-activated transcription factors, which also include receptors for non-steroids such as thyroid hormone, retinoids and vitamin D, as well as orphan receptors whose ligands and/or functions are unknown (Weigel, 1996). In the absence of ligands, steroid receptors are associated with heat shock proteins (HSPs) of 90 kDa and 70 kDa (Smith, 1993). Engagement of the receptors to steroids leads to the dissociation of the HSPs and receptor dimerization (Figure 1.4). As a result, the dimerized receptor binds to

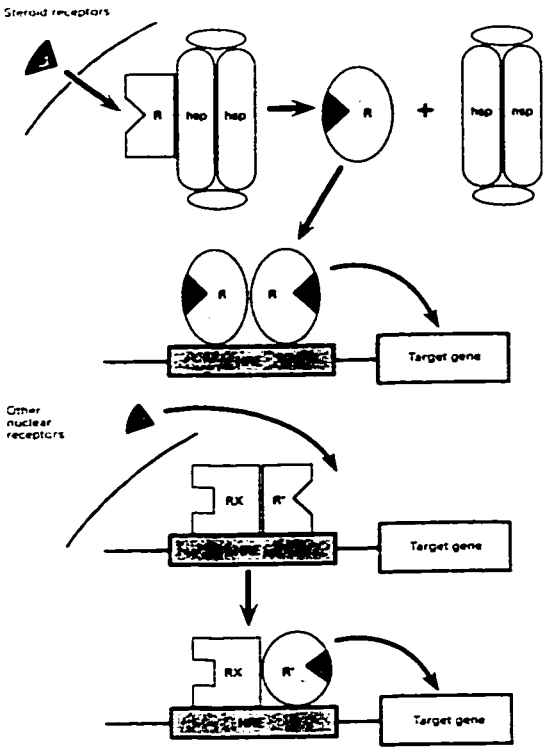


Figure 1.4 Action of steroid/thyroid hormone receptors (Weigel, 1996). Upper panel depicts activation of steroid receptors. In the absence of steroids, each receptor (R) monomer is associated with a HSP complex. Binding of steroids (S) causes a conformational change and results in dissociation of the HSP complex and receptor dimerization. Consequently, dimerized receptors bind to a hormone response element (HRE) of the target gene and activate the transcription of the gene. Lower panel describes the action of non-steroid receptors (R'). In the absence of ligands, R' typically forms a heterodimer with retinoid X receptor (RXR), which binds to HRE of the target gene and repress the transcription of the gene. Binding of hormone (H) activates the receptor and causes the activation of gene transcription.

a specific steroid response element of target genes, and subsequently interacts with basal transcription factors, other DNA binding proteins or co-activators, resulting in transcription of these genes (Weigel, 1996; Ing et al., 1992; Schule et al., 1988a and 1988b). In contrast, the receptors in this family for non-steroids typically form heterodimers with retinoid X receptors (RXRs) in the nucleus, where they are tightly bound to specific response elements of target genes in the absence of ligands and repress the transcription of these genes (Mangelsdorf et al., 1990; Leid et al., 1992; Yu et al., 1991) (Fig. 1.4). Ligand binding may cause dissociation of repressor molecules and allow activation of transcription of target genes (Weigel, 1996; Horlein et al., 1995; Kurokawa et al., 1995).

B. Signal Transduction via Cell Surface Receptors

Engagement of cell surface receptors for growth factors and cytokines initiates a cascade of signal events, which causes complex cytoplasmic and nuclear responses. There are many signal transduction pathways mediated by different types of receptors. Here are a few major pathways possibly involved in mammalian implantation.

1. RAS-MAPK pathway

Many receptors for cytokines and growth factors contain an intracellular protein tyrosine kinase (PTK) or associate with non-receptor PTKs containing SH2 and SH3 domains. Ligand binding induces the receptor to dimerize, which activates the kinase activity of either the receptor or its associated PTK (Heldin, 1995 and 1996). When activated, receptor tyrosine kinases usually cross-phosphorylate themselves on multiple tryosine residues, which then serve as docking sites for downstream signaling proteins.

Table 1. 6 Ligands activating the JAK and STAT pathway

Family	Members
IFN	IFNα, IFNγ, IL-10
Receptor tyrosine kinases	EGF, CSF-1, SCF, PDGF
gp130	LIF, IL-6, IL-11, IL-12, CNTF, G-CSF, OnM
gp140	IL-3, IL-5, GM-CSF
Seven transmembrane receptor	Angiotensin II
IL-2	IL-2, -4, -7, -9, -13, -15
Growth hormone (GH)	GH, prolactin, erythropoietin, thrombopoietin

Ras protein is active when bound to GTP and inactive when bound to GDP. The activation state of Ras protein is regulated by GTPase activating protein (GAP) (negative regulator) and guanine nucleotide exchange factor (Sos) (positive regulator) (Lowy and Willumsen, 1993). Autophosphorylated tyrosine kinase receptors could recruit and activate the complex of adaptor protein Grb2 and Sos directly (or through another adaptor Shc). This causes translocation of Sos to the plasma membrane where Ras is located, and hence increases the rate of nucleotide exchange on Ras leading to its activation (Downward, 1996). Subsequently, activated Ras triggers an activation cascade of serine/threonine kinases including Raf-1, MEK (MAPKK) and MAPK. Activated MAPK is translocated into the nucleus, where it phosphorylates certain transcription factors. This pathway is important for stimulation of cell growth.

2. The JAK-STAT pathway

Molecular characterization of IFN-induced gene activation led to the discovery of the JAK-STAT signal transduction pathway, which is now known to be shared by other cytokines, growth factors and hormones (Williams and Haque, 1997; Shauai, 1994; Ihle et al., 1994). Binding of these ligands to their transmembrane receptors result in activation of members of the Janus family tyrosine kinase (JAK). The activated receptor-kinase complexes recruit members of the signal transducers and activators of transcription (STAT). As a consequence, the phosphorylated STAT proteins dimerize, translocate into the nucleus, bind response elements of target genes and activate the transcription of these genes. The specificity of biological effects is determined by the specific activation of members of the JAK and STAT families by the ligand. The ligands identified to activate the JAK and STAT pathway are listed in Table 1.6 (Heim, 1996).

3. Integrin signal pathway

Integrin-mediated signaling is also a complex process that shares some characteristics with those of growth factors and cytokines, including receptor cross-linking, up-regulation of tyrosine phosphorylation and activation of MAPK (Yurochko, 1997). However, integrin engagement causes the phosphorylation of a non-receptor tyrosine kinase named focal adhesion kinase (FAK), which may activate other SH2-containing kinases including Src, Fyn and phosphatidyinositol 3-kinase. Adhesion-induced activation of MAPK and the association of Grb-2 with FAK suggest that integrins might activate the Ras-MAPK signal pathway (Chen et al., 1994; Schlaepfer et al., 1994). However, integrin signaling to MAPK seems to be independent of Ras (Juliano, 1996).

In addition, the cytoskeleton could play a role in most aspects of integrin signaling since it is associated with integrins and FAK at focal contacts, which may be a mechanism for localizing some of the important factors in the signal transduction cascade (Yurochko, 1997; Juliano, 1996). Furthermore, other transmembrane proteins such as TM4 superfamily proteins (CD9, CD63 and CD81) are also involved in integrin signaling (Berditchevski et al., 1997; Shaw et al., 1995). It is clear that there is a lot about integrin signaling that remains to be worked out.

OBJECTIVES OF THE PRESENT STUDY

In the pig, embryonic mortality within the first 30 days of gestation could reduce potential litter size by 30 to 40%, and considerable losses occur from Days 13 to 20, coincident with blastocyst elongation and implantation (attachment). Early embryogenesis and implantation are two critical processes that occur during the peri-implantation period in the pig. To date, little is known about the mechanisms regulating early embryogenesis and implantation in the pig. Understanding these mechanisms might allow insights to aberrant processes of embryogenesis that may contribute to embryonic death in this important domestic animal. The overall objective of this study was to characterize the gene expression of important protein factors in blastocysts and the endometrium during the peri-implantation period.

OBJECTIVE 1 To determine the identity of Jag-1 macrophage growth factor (MGF) and to characterize the expression of this growth factor (s) in blastocysts and the endometrium during pre-implantation development in the pig.

Rationale Growth factors/cytokines are a group of protein factors, which are directly involved in the communication between the embryo and the endometrium during early mammalian embryo development (Simon et al., 1998; Schultz and Heyner, 1993). They have also been demonstrated to be important cellular regulators for MMP, TIMP and integrin production and activity *in vitro* (Sehgal and Thompson, 1999; Bischof et al., 1998; Parsons and Parsons, 1997; Harvey et al., 1995b). A trophoblast cell line, Jag-1, was previously isolated from Day-14 porcine embryos and was shown to produce growth factor (s) that could stimulate the proliferation of porcine macrophages *in vitro* (Ramsoondar et al., 1993). Upon reaching a size of about 10mm in diameter on Day 10 of pregnancy, porcine blastocysts undergo extensive elongation (Geisert and Yelich, 1997). This elongation process requires both the rapid proliferation of the trophoblast and cellular reorganization (Geisert et al., 1982b). Therefore, characterization of the macrophage growth factor (s) might help us understand the blastocyst elongation process. A previous study by Ramsoondar (1994) demonstrated that a protein species with a size of approximately 40 kDa was associated with the bioactivity and a strong hybridization signal to Jag-1 cellular RNA was obtained by Northern blot analysis with a probe for human CSF-1 (36-45 kDa). Therefore, **the hypothesis for the first experiment** of the present study was that **CSF-1 was the candidate for Jag-1 macrophage growth factor (MGF)**. This subject is addressed in Chapter 2.

Experimental approach 1) Examine the inhibitory effect of an anti-human CSF-1 monoclonal antibody on the proliferation of porcine macrophages by the Jag-1 MGF. 2) Clone the cDNA sequence of the MGF from Jag-1 cellular RNA. 3) Determine the copy number of the MGF gene in the porcine genome. 4) Examine the expression of the MGF in blastocysts and the endometrium during pre-implantation development in the pig using a RT-PCR approach.

OBJECTIVE 2 Mechanism regulating embryo implantation in the pig.

Rationale Embryo implantation during pregnancy in pigs is a non-invasive process that results in simple attachment between the trophoblast and the uterine epithelium. This type of placentation is termed epitheliochorial to distinguish it from other more invasive types of placentation, such as the hemochorial placentation seen in rodents and humans. However, when porcine embryos were artificially transplanted to ectopic sites, the trophoblast cells appeared to be highly invasive (Samuel and Perry, 1972a&b). This suggests that either the environmental cues present at the ectopic site caused these cells to become invasive or, alternatively, that the intrinsically invasive nature of these cells must be regulated *in vivo* during normal conceptus development. **The hypothesis for the second experiment** of the present study was that **the balance between ECM degradation and deposition in the embryo and at the interface between the trophoblast and the uterine epithelium must be tightly controlled**, during the peri-implantation period, such that implantation and early embryogenesis can proceed appropriately. This subject is addressed in Chapter 3.

Experimental approach 1) Characterize the temporal expression of MMP, TIMP and uPA transcripts in blastocysts and the endometrium during the peri-implantation period using a RT-PCR approach. 2). Examine the presence of proMMPs and TIMPs in uterine fluids prior to implantation using zymography and reverse zymography techniques.

OBJECTIVE 3 To examine the expression of integrin transcripts in blastocysts and in the endometrium during the peri-implantation in the pig.

Rationale Early embryogenesis and implantation involve extensive cell-cell and cell-extracellular matrix interactions, which are mediated through adhesion molecules that include Muc-1, heparin sulfate proteoglycans, lectins, cadherins, ECM components, and integrins (Aplin, 1997). Integrins are critical for signal transduction from the ECM into the cell and play an important role during early embryogenesis, implantation and placentation in the mouse (Coutifaris et al., 1998; Damsky et al., 1997). The presence of some integrin subunits was demonstrated in previous studies by Bowen et al. (1996 & 1997). **The hypothesis for the third experiment** of the present study was that **the expression of integrins must be developmentally regulated in blastocysts and the endometrium during the peri-implantation period** in order to facilitate cell migration during extraembryonic endodermal and mesodermal formation, and the attachment between the trophoblast and the uterine epithelium during implantation in the pig. This subject is addressed in Chapter 4.

Experimental approach Examine the expression of integrin transcripts in blastocysts and the endometrium during the peri-implantation in the pig using a RT-PCR approach.

REFERENCES

Abbondanzo SJ. Cullinan EB. McIntyre K. Labow MA. Stewart CL. Reproduction in mice lacking a functional type 1 IL-1 receptor. Endocrinology. 137(8):3598-601, 1996

Adler RR. Brenner CA. Werb Z. Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonic carcinoma cells. Development. 110(1):211-20, 1990

Agura ED. Howard M. Collins SJ. Identification and sequence analysis of the promoter for the leukocyte integrin beta-subunit (CD18): a retinoic acid-inducible gene. Blood. 79(3):602-9, 1992

Alexander CM. Hansell EJ. Behrendtsen O. Flannery ML. Kishnani NS. Hawkes SP. Werb Z. Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. Development. 122(6):1723-36, 1996

Amoroso EC. Placentation. In Marshall's Physiology of Reproduction, Vol. 2 (3rd edition), ed. by A.S. Parkes. Longman, Green and Co., pp. 127-311, 1952.

Anderson LH. Christenson LK. Christenson RK. Ford SP. Investigations into the control of litter size in swine: II. Comparisons of morphological and functional embryonic diversity between Chinese and American breeds. Journal of Animal Science. 71(6):1566-71, 1993

Anderson LL. Growth, protein content and distribution of early pig embryos. Anatomical Record. 190(1):143-53, 1978

Anegon I. Cuturi MC. Godard A. Moreau M. Terqui M. Martinat-Botte F. Soulillou JP. Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. Cytokine. 6(5):493-9, 1994

Aplin JD. Adhesion molecules in implantation. Reviews of Reproduction. 2(3):84-93, 1997

Arceci RJ. Shanahan F. Stanley ER. Pollard JW. Temporal expression and location of colony-stimulating factor 1 (CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1-regulated placental development. PNAS USA. 86(22):8818-22, 1989

Barends PM. Stroband HW. Taverne N. Kronnie G. Leen MP. Blommers PC. Integrity of the preimplantation pig blastocyst during expansion and loss of polar trophectoderm (Rauber cells) and the morphology of the embryoblast as an indicator for developmental stage. Journal of Reproduction & Fertility. 87(2):715-26, 1989

Bazer FW. Geisert RD. Thatcher WW. Roberts RM. The establishment and maintenance of pregnancy. In Control of Pig Reproduction, ed. by D.J.A. Cole and G.R. Foxcroft. Butterworths, London, UK. Pg: 227-252, 1982

Bazer FW. New frontiers and approaches in the study of embryonic mortality in domestic species. In Embryonic Mortality in Domestic Species, ed. by Michael T. Zavy and Rodney D. Geisert, CRC Press, Boca Raton, Florida, USA. Pg. 195-210, 1994a

Bazer FW. Ott TL. Spencer TE. Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology 41:79-94, 1994b

Bazer FW. Simmen RC. Simmen FA. Comparative aspects of conceptus signals for maternal recognition of pregnancy. Annals of the New York Academy of Sciences. 622: 202-11, 1991

Bazer FW. Spencer TE. Ott TL. Interferon tau: a novel pregnancy recognition signal. American Journal of Reproductive Immunology. 37(6):412-20, 1997

Bazer FW. Spencer TE. Ott TL. Placental interferons. American Journal of Reproductive Immunology. 35(4):297-308, 1996

Bazer FW. Vallet JL. Harney JP. Gross TS. Thatcher WW. Comparative aspects of maternal recognition of pregnancy between sheep and pigs. Journal of Reproduction & Fertility - Supplement. 37:85-9, 1989

Bazer FW. Vallet JL. Roberts RM. Sharp DC. Thatcher WW. Role of conceptus secretory products in establishment of pregnancy. Journal of Reproduction & Fertility. 76(2):841-50, 1986

Behrendtsen O. Alexander CM. Werb Z. Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development. 114(2):447-56, 1992

Berditchevski F. Tolias KF. Wong K. Carpenter CL. Hemler ME. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. Journal of Biological Chemistry. 272(5):2595-8, 1997

Bhatt H. Brunet LJ. Stewart CL . Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. PNAS USA. 88(24):11408-12, 1991

Bhoola KD. Figueroa CD. Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacological Reviews. 44(1):1-80, 1992

Biggers JD. Mammalian blastocyst and amnion formation. In The Water Metabolism of the Fetus, ed. by A.C. Barnes. Springfield. pp. 3-31, 1972

Bischof P. Meisser A. Campana A. Tseng L. Effects of decidua-conditioned medium and insulin-like growth factor binding protein-1 on trophoblastic matrix metalloproteinases and their inhibitors. Placenta. 19(7):457-64, 1998

Bishop MWH. Prenatal mortality in farm animals. Journal of Reproduction and Fertility. 2:182-94, 1961.

Blomhoff R. Green MH. Berg T. Norum KR. Transport and storage of vitamin A. Science. 250(4979):399-404, 1990

Boice ML. Geisert RD. Blair RM. Verhage HG. Identification and characterization of bovine oviductal glycoproteins synthesized at estrus. Biology of Reproduction. 43(3):457-65, 1990

Boomsma RA. Mavrogianis PA. Verhage HG. Immunocytochemical localization of transforming growth factor alpha, epidermal growth factor and epidermal growth factor receptor in the cat endometrium and placenta. Histochemical Journal. 29(6):495-504, 1997

Bowen JA. Bazer FW. Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biology of Reproduction. 56(2):409-15, 1997

Bowen JA. Bazer FW. Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biology of Reproduction. 55(5):1098-106, 1996

Brenner CA. Adler RR. Rappolee DA. Pedersen RA. Werb Z . Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes & Development. 3(6):848-59, 1989

Brigstock DR. Kim GY. Steffen CL. Liu A. Vegunta RK. Ismail NH. High molecular mass forms of epidermal growth factor in pig uterine secretions. Journal of Reproduction & Fertility. 108(2):313-20, 1996

Burghardt RC. Bowen JA. Newton GR. Bazer FW. Extracellular matrix and the implantation cascade in pigs. Journal of Reproduction & Fertility - Supplement. 52:151-64, 1997.

Carmeliet P. Schoonjans L. Kieckens L. Ream B. Degen J. Bronson R. De Vos R. van den Oord JJ. Collen D. Mulligan RC. Physiological consequences of loss of plasminogen activator gene function in mice. Nature. 368(6470):419-24, 1994

Cary LA. Guan JL. Focal adhesion kinase in integrin-mediated signaling. Frontiers in Bioscience. 4:D102-13, 1999.

Chamberlin R. Menino AR. Partial characterization of gelatinases produced by preimplantation porcine embryos. Biology of Reproduction. 52(Suppl):179, 1995

Chastant S. Monget P. Terqui M. Localization and quantification of insulin-like growth factor-I (IGF-I) and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors in pig embryos during early pregnancy. Biology of Reproduction. 51(4):588-96, 1994

Chen Q. Kinch MS. Lin TH. Burridge K. Juliano RL. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. Journal of Biological Chemistry. 269(43):26602-5, 1994

Chia CM. Winston RM. Handyside AH. EGF, TGF-alpha and EGFR expression in human preimplantation embryos. Development. 121(2):299-307, 1995

Chiocca EA. Davies PJ. Stein JP. Regulation of tissue transglutaminase gene expression as a molecular model for retinoid effects on proliferation and differentiation. Journal of Cellular Biochemistry. 39(3):293-304, 1989

Choudhuri R. Wood GW . Production of interleukin-1, interleukin-6, and tumor necrosis factor alpha in the uterus of pseudopregnant mice. Biology of Reproduction. 49(3):596-603, 1993

Clawitter J. Trout WE. Burke MG. Araghi S. Roberts RM. A novel family of progesterone-induced, retinol-binding proteins from uterine secretions of the pig. Journal of Biological Chemistry. 265(6):3248-55, 1990

Cohen PE, Zhu L, Pollard JW (1997): Absence of colony-stimulating factor-1 in osteopetrotic ($csfm^{op}/csfm^{op}$) mice disrupts estrous cycles and ovulation. Biol. Reprod. 56: 110-118.

Conley AJ. Christenson LK. Ford SP. Christenson RK. Immunocytochemical localization of cytochromes P450 17 alpha-hydroxylase and aromatase in embryonic cell layers of elongating porcine blastocysts. Endocrinology. 135(6):2248-54, 1994

Conley AJ. Christenson RK. Ford SP. Geisert RD. Mason JI. Steroidogenic enzyme expression in porcine conceptuses during and after elongation. Endocrinology. 131(2):896-902, 1992

Coutifaris C. Omigbodun A. Coukos G. Integrins, endometrial maturation, & human embryo implantation. Seminars In Reproductive Endocrinology. 16(3):219-29, 1998

Cross JC. Roberts RM. Porcine conceptuses secrete an interferon during the preattachment period of early pregnancy. Biology of Reproduction. 40(5):1109-18, 1989

Cross JC. Werb Z. Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science. 266(5190):1508-18, 1994

Damsky C. Schick SF. Klimanskaya I. Stephens L. Zhou Y. Fisher S. Adhesive interactions in peri-implantation morphogenesis and placentation. Reproductive Toxicology. 11(2-3):367-75, 1997

Damsky C. Sutherland A. Fisher S. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. FASEB Journal. 7(14):1320-9, 1993

Dantzer V. Bjorkman N. Hasselager E. An electron microscopic study of histiotrophe in the interareolar part of the porcine placenta. Placenta. 2(1):19-28, 1981

Dantzer V. Electron microscopy of the initial stages of placentation in the pig. Anatomy & Embryology. 172(3):281-93, 1985

Das SK. Lim H. Wang J. Paria BC. BazDresch M. Dey SK. Inappropriate expression of human transforming growth factor (TGF)-alpha in the uterus of transgenic mouse causes downregulation of TGF-beta receptors and delays the blastocyst-attachment reaction. Journal of Molecular Endocrinology. 18(3):243-57, 1997

Das SK. Chakraborty I. Paria BC. Wang XN. Plowman G. Dey SK. Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. Molecular Endocrinology. 9(6):691-705, 1995

Das SK. Stanley ER . Structure-function studies of a colony stimulating factor (CSF-1). Journal of Biological Chemistry. 257(22):13679-84, 1982

Day BN. Anderson LL. Emmerson MA. Hazel LN. Melampy RM. Effect of estrogen and progesterone on early embryonic mortality in ovariectomized gilts. Journal of Animal Science. 18:607-613, 1959.

De los Santos MJ. Mercader A. Frances A. Portoles E. Remohi J. Pellicer A. Simon C. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. Biology of Reproduction. 54(3):563-74, 1996

De Luca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB Journal. 5(14):2924-33, 1991

De M. Sanford TR. Wood GW . Expression of interleukin 1, interleukin 6 and tumour necrosis factor alpha in mouse uterus during the peri-implantation period of pregnancy. Journal of Reproduction & Fertility. 97(1):83-9, 1993

Dhindsa DS. Dziuk PJ. Influence of varying the proportion of uterus occupied by embryos on maintenance of pregnancy in the pig. Journal of Animal Science. 27(3):668-72, 1968

Dhindsa DS. Dziuk PJ. Norton HW. Time of transuterine migration and distribution of embryos in the pig. Anatomical Record. 159(3):325-30, 1967

Diederich MJ. Yelich JV. Geisert RD. Gene and protein expresssion of the inter-trypsin inhibitor, bikunin, in the porcine conceptus and endometrium. Journal of Animal Science. 75 (Supplement 1):83, 1997.

Downward J. Control of Ras activation. In Cell Signalling, ed. by P.J. Parker and T. Pawson. Cold Spring Harbor Laboratory Press, New York. Pp.87-100, 1996

Dower SK. Kronheim SR. Hopp TP. Cantrell M. Deeley M. Gillis S. Henney CS. Urdal DL . The cell surface receptors for interleukin-1 alpha and interleukin-1 beta are identical. Nature. 324(6094):266-8, 1986

Duffy JY. Stallings-Mann ML. Trout WE. Roberts RM. Expression of a plasmin/trypsin Kunitz inhibitor by pig trophoblast. Molecular Reproduction & Development. 46(4):443-9, 1997

Dziuk PJ. Embryonic loss in the pig: an enigma. In Manipulating Pig Production, ed. by Barnett JL et al., Australian Pig Science Association, Victoria, Austrlia. Pg: 28-39, 1987

Elangovan S. Moulton BC. Blastocyst implantation in the rat and the immunohistochemical distribution and rate of synthesis of uterine lysosomal cathepsin D. Biology of Reproduction. 23(3):663-8, 1980

Fazleabas AT. Geisert RD. Bazer FW. Roberts RM. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. Biology of Reproduction. 29(1):225-38, 1983

Fischer HE. Bazer FW. Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. Journal of Reproduction

Fertility. 75(1):69-78, 1985

Flint AP. A unifying hypothesis for the control of blastocyst growth based on observations of the pig. Journal of Reproduction & Fertility - Supplement. 29:215-27, 1981

Flood PF. The development of the conceptus and its relationship to the uterus. In Reproduction in Domestic Animals, (4th edition), ed. by P.T. Cupps. Academic Press, pp. 315-360, 1991.

Ford SP. Youngs CR. Early embryonic development in prolific Meishan pigs. Journal of Reproduction & Fertility - Supplement. 48:271-8, 1993.

Ford SP. Christenson RK. Ford JJ. Uterine blood flow and uterine arterial, venous and luminal concentrations of oestrogens on days 11, 13 and 15 after oestrus in pregnant and non-pregnant sows. Journal of Reproduction & Fertility. 64(1):185-90, 1982

Fortunato SJ. Menon R. Lombardi SJ. Presence of four tissue inhibitors of matrix metalloproteinases (TIMP-1, -2, -3 and -4) in human fetal membranes. American Journal of Reproductive Immunology. 40(6):395-400, 1998

Foxcroft GR. Mechanisms mediating nutritional effects on embryonic survival in pigs. Journal of Reproduction & Fertility - Supplement. 52:47-61, 1997

Gadsby JE. Heap RB. Burton RD. Oestrogen production by blastocyst and early embryonic tissue of various species. Journal of Reproduction & Fertility. 60(2):409-17, 1980

Geisert RD. Yelich JV. Regulation of conceptus development and attachmen in pigs. Journal of Reproduction & Fertility - Supplement. 52:133-149, 1997.

Geisert RD. Pratt TN. Bazer FW. Mayes JS. Watson GH. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reproduction, Fertility, & Development. 6(6):749-60, 1994a

Geisert RD. Short EC. Morgan GL. Establishment of pregnancy in domestic farm species. In Embryonic Mortality in Domestic Species, ed. by Michael T. Zavy and Rodney D. Geisert, CRC Press, Florida, USA. Pg. 23-51, 1994b.

Geisert RD. Brenner RM. Moffatt RJ. Harney JP. Yellin T. Bazer FW. Changes in oestrogen receptor protein, mRNA expression and localization in the endometrium of cyclic and pregnant gilts. Reproduction, Fertility, & Development. 5(3):247-60, 1993

Geisert RD. Morgan GL. Short EC Jr. Zavy MT. Endocrine events associated with endometrial function and conceptus development in cattle. Reproduction, Fertility, & Development. 4(3):301-5, 1992

Geisert RD. Morgan GL. Zavy MT. Blair RM. Gries LK. Cox A. Yellin T. Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos. Journal of Reproduction & Fertility. 93(2):475-81, 1991

Geisert RD. Zavy MT. Moffatt RJ. Blair RM. Yellin T. Embryonic steroids and the establishment of pregnancy in pigs. Journal of Reproduction & Fertility - Supplement. 40:293-305, 1990

Geisert RD. Renegar RH. Thatcher WW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biology of Reproduction. 27(4):925-39, 1982a

Geisert RD. Brookbank JW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biology of Reproduction. 27(4):941-55, 1982b

Geisert RD. Thatcher WW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. Biology of Reproduction. 27(4):957-65, 1982c

Gharib-Hamrouche N. Chene N. Martal J. Comparative expression of TGF-alpha and EGF genes in the ovine conceptus and uterine endometrium in the peri-implantation period. Reproduction, Nutrition, Development. 35(3):291-303, 1995

Gille J. Swerlick RA. Integrins: role in cell adhesion and communication. Annals of the New York Academy of Sciences. 797:93-106, 1996

Godkin JD. Bazer FW. Lewis GS. Geisert RD. Roberts RM. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biology of Reproduction. 27(4):977-87, 1982

Graham CH. Connelly I. MacDougall JR. Kerbel RS. Stetler-Stevenson WG. Lala PK. Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. Experimental Cell Research. 214(1):93-9, 1994

Green ML. Simmen RC. Simmen FA. Developmental regulation of steroidogenic enzyme gene expression in the periimplantation porcine conceptus: a paracrine role for insulin-like growth factor-I. Endocrinology. 136(9):3961-70, 1995

Guillomot M. Flechon JE. Wintenberger-Torres S. Conceptus attachment in the ewe: an ultrastructural study. Placenta. 2(2):169-82, 1981

Gupta A. Bazer FW. Jaeger LA. Differential expression of beta transforming growth factors (TGF beta 1, TGF beta 2, and TGF beta 3) and their receptors (type I and type II) in peri-implantation porcine conceptuses. Biology of Reproduction. 55(4):796-802, 1996

Hamilton JA. CSF-1 and cell cycle control in macrophages. Molecular Reproduction & Development. 46(1):19-23, 1997

Harney JP. Ali M. Vedeckis WV. Bazer FW. Porcine conceptus and endometrial retinoid-binding proteins. Reproduction, Fertility, & Development. 6(2):211-9, 1994a

Harney JP. Ott TL. Geisert RD. Bazer FW. Retinol-binding protein gene expression in cyclic and pregnant endometrium of pigs, sheep, and cattle. Biology of Reproduction. 49(5):1066-73, 1993

Harney JP. Smith LC. Simmen RC. Fliss AE. Bazer FW. Retinol-binding protein: immunolocalization of protein and abundance of messenger ribonucleic acid in conceptus and maternal tissues during pregnancy in pigs. Biology of Reproduction. 50(5):1126-35, 1994b

Harvey MB. Leco KJ. Arcellana-Panlilio MY. Zhang X. Edwards DR. Schultz GA. Roles of growth factors during peri-implantation development. Human Reproduction. 10(3):712-8, 1995a

Harvey MB. Leco KJ. Arcellana-Panlilio MY. Zhang X. Edwards DR. Schultz GA. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Development. 121(4):1005-14, 1995b

Hauser H. Interferons. In Growth Factors, Differentiation Factors, and Cytokines, ed. by A. Habenicht, Springer-Verlag, Berlin, Germany. pp: 243-253, 1990.

Hearn JP. Webley GE. Gidley-Baird AA. Chorionic gonadotrophin and embryo-maternal recognition during the peri-implantation period in primates. Journal of Reproduction & Fertility. 92(2):497-509, 1991

Heldin CH. Prtoein tyrosine kinase receptors. In Cell Signalling, ed. by P.J. Parker and T. Pawson. Cold Spring Harbor Laboratory Press, New York. Pp.7-40, 1996

Heldin CH. Dimerization of cell surface receptors in signal transduction. Cell. 80(2):213-23, 1995

Heim MH. The Jak-STAT pathway: specific signal transduction from the cell membrane to the nucleus. European Journal of Clinical Investigation. 26:1-12, 1997.

Heldin CH. Dimerization of cell surface receptors in signal transduction. Cell. 80:213-223, 1995

Hemler ME. Integrin associated proteins. Current Opinion in Cell Biology. 10(5):578-85, 1998.

Herrmann BG. Labeit S. Poustka A. King TR. Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse. Nature. 343(6259):617-22, 1990

Hofig A. Michel FJ. Simmen FA. Simmen RC. Constitutive expression of uterine receptors for insulin-like growth factor-I during the peri-implantation period in the pig. Biology of Reproduction. 45(4):533-9, 1991a

Hofig A. Simmen FA. Bazer FW. Simmen RC. Effects of insulin-like growth factor-I on aromatase cytochrome P450 activity and oestradiol biosynthesis in preimplantation porcine conceptuses in vitro. Journal of Endocrinology. 130(2):245-50, 1991b

Horlein AJ. Naar AM. Heinzel T. Torchia J. Gloss B. Kurokawa R. Ryan A. Kamei Y. Soderstrom M. Glass CK. et al. Ligand-independent repression by the thyroid

hormone receptor mediated by a nuclear receptor co-repressor. Nature. 377(6548):397-404, 1995

Hu XL. Yang Y. Hunt JS . Differential distribution of interleukin-1 alpha and interleukin-1 beta proteins in human placentas. Journal of Reproductive Immunology. 22(3):257-68, 1992

Hunter RHF. Chronological and cytological details of fertilization and early embryonic development in the domestic pig, *Sus scrofa*. Anatomical Record 178:169-86, 1974.

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 69(1):11-25, 1992

Ihle JN. Witthuhn BA. Quelle FW. Yamamoto K. Thierfelder WE. Kreider B. Silvennoinen O. Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends in Biochemical Sciences. 19(5):222-7, 1994

Ing NH. Beekman JM. Tsai SY. Tsai MJ. O'Malley BW. Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). Journal of Biological Chemistry. 267(25):17617-23, 1992

Ishikawa T. Umesono K. Mangelsdorf DJ. Aburatani H. Stanger BZ. Shibasaki Y. Imawari M. Evans RM. Takaku F. A functional retinoic acid receptor encoded by the gene on human chromosome 12. Molecular Endocrinology. 4(6):837-44, 1990

Jetten AM. Retinoids specifically enhance the number of epidermal growth factor receptors. Nature. 284(5757):626-9, 1980

Johnson DC. A phorbol ester that activates protein kinase C mimics the action of estradiol or epidermal growth factor for initiating embryo implantation in the delayed implanting hypophysectomized rat. Life Sciences. 58(10):861-7, 1996

Jokhi PP. Chumbley G. King A. Gardner L. Loke YW . Expression of the colony stimulating factor-1 receptor (c-fms product) by cells at the human uteroplacental interface. Laboratory Investigation. 68(3):308-20, 1993

Jokhi PP. King A. Boocock C. Loke YW . Secretion of colony stimulating factor-1 by human first trimester placental and decidual cell populations and the effect of this cytokine on trophoblast thymidine uptake in vitro. Human Reproduction. 10(10):2800-7, 1995

Juliano R. Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. Bioessays. 18(11):911-7, 1996

Kauma SW. Aukerman SL. Eierman D. Turner T . Colony-stimulating factor-1 and c-fms expression in human endometrial tissues and placenta during the menstrual cycle and early pregnancy. Journal of Clinical Endocrinology & Metabolism. 73(4):746-51, 1991

Kauma SW. Interleukin-1 beta stimulates colony-stimulating factor-1 production in human term placenta. Journal of Clinical Endocrinology & Metabolism. 76(3):701-3, 1993

Kaye PL. Preimplantation growth factor physiology. Reviews of Reproduction. 2(2):121-7, 1997

Kaye PL. Harvey MB. The role of growth factors in preimplantation development. Progress in Growth Factor Research. 6(1):1-24, 1995

Kemp B. Soede NM. Consequences of variation in interval from insemination to ovulation on fertilization in pigs. Journal of Reproduction & Fertility - Supplement. 52:79-89, 1997

Kennedy TG. Brown KD. Vaughan TJ. Expression of the genes for the epidermal growth factor receptor and its ligands in porcine oviduct and endometrium. Biology of Reproduction. 50(4):751-6, 1994

Keys JL. King GJ. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. American Journal of Anatomy. 188(3):221-38, 1990

Kim GY. Besner GE. Steffen CL. McCarthy DW. Downing MT. Luquette MH. Abad MS. Brigstock DR. Purification of heparin-binding epidermal growth factor-like growth factor from pig uterine luminal flushings, and its production by endometrial tissues. Biology of Reproduction. 52(3):561-71, 1995

Kimelman D. Kirschner M. Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell. 51(5):869-77, 1987

King A. Jokhi PP. Smith SK. Sharkey AM. Loke YW. Screening for cytokine mRNA in human villous and extravillous trophoblasts using the reverse-transcriptase polymerase chain reaction (RT-PCR). Cytokine. 7(4):364-71, 1995

Kishimoto T. Akira S. Taga T. IL-6 receptor and mechanism of signal transduction. International Journal of Immunopharmacology. 14(3):431-8, 1992

Ko Y. Choi I. Green ML. Simmen FA. Simmen RC. Transient expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development. Molecular Reproduction & Development. 37(1):1-11, 1994

Kruessel JS. Huang HY. Wen Y. Kloodt AR. Bielfeld P. Polan ML. Different pattern of interleukin-1 beta-(IL-1 beta), interleukin-1 receptor antagonist- (IL-1ra) and interleukin-1 receptor type I- (IL-1R tI) mRNA-expression in single preimplantation mouse embryos at various developmental stages. Journal of Reproductive Immunology. 34(2):103-20, 1997

Kurokawa R. Soderstrom M. Horlein A. Halachmi S. Brown M. Rosenfeld MG. Glass CK. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature. 377(6548):451-4, 1995

Laird SM. Tuckerman E. Li TC. Bolton AE. Stimulation of human endometrial epithelial cell interleukin 6 production by interleukin 1 and placental protein 14. Human Reproduction. 9(7):1339-43, 1994

Lammer EJ. Chen DT. Hoar RM. Agnish ND. Benke PJ. Braun JT. Curry CJ. Fernhoff PM. Grix AW Jr. Lott IT. Retinoic acid embryopathy. New England Journal of Medicine. 313(14):837-41, 1985

Lasky LA. Selectin-carbohydrate interactions and the initiation of the inflammatory response. Annual Review of Biochemistry. 64:113-39, 1995

Leco KJ. Apte SS. Taniguchi GT. Hawkes SP. Khokha R. Schultz GA. Edwards DR. Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. FEBS Letters. 401(2-3):213-7, 1997

Leco KJ. Edwards DR. Schultz GA. Tissue inhibitor of metalloproteinases-3 is the major metalloproteinase inhibitor in the decidualizing murine uterus. Molecular Reproduction & Development. 45(4):458-65, 1996

Leid M. Kastner P. Lyons R. Nakshatri H. Saunders M. Zacharewski T. Chen JY. Staub A. Garnier JM. Mader S. et al. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell. 68(2):377-95, 1992

Lessey BA. Castelbaum AJ. Buck CA. Lei Y. Yowell CW. Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertility & Sterility. 62(3):497-506, 1994

Lessey BA. Damjanovich L. Coutifaris C. Castelbaum A. Albelda SM. Buck CA. Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. Journal of Clinical Investigation. 90(1):188-95, 1992

Lessey BA. Yeh I. Castelbaum AJ. Fritz MA. Ilesanmi AO. Korzeniowski P. Sun J. Chwalisz K. Endometrial progesterone receptors and markers of uterine receptivity in the window of implantation. Fertility & Sterility. 65(3):477-83, 1996

Letcher R. Simmen RC. Bazer FW. Simmen FA. Insulin-like growth factor-I expression during early conceptus development in the pig. Biology of Reproduction. 41(6):1143-51, 1989

Lewis GS. Waterman RA. Metabolism of arachidonic acid in vitro by porcine blastocysts and endometrium. Prostaglandins. 25(6):871-80, 1983

Li W. Jaffe RC. Verhage HG. Immunocytochemical localization and messenger ribonucleic acid levels of a progesterone-dependent endometrial secretory protein (cathepsin L) in the pregnant cat uterus. Biology of Reproduction. 47(1):21-8, 1992

Librach CL. Werb Z. Fitzgerald ML. Chiu K. Corwin NM. Esteves RA. Grobelny D. Galardy R. Damsky CH. Fisher SJ. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. Journal of Cell Biology. 113(2):437-49, 1991

Lim H. Dey SK. Das SK. Differential expression of the erbB2 gene in the periimplantation mouse uterus: potential mediator of signaling by epidermal growth factor-like growth factors. Endocrinology. 138(3):1328-37, 1997

Linney E. Retinoic acid receptors: transcription factors modulating gene regulation, development, and differentiation. Current Topics in Developmental Biology. 27:309-50, 1992

Lowy DR. Willumsen BM. Function and regulation of RAS. Annual Review of Biochemistry. 62:851-891, 1993

Malathy PV. Imakawa K. Simmen RC. Roberts RM. Molecular cloning of the uteroferrin-associated protein, a major progesterone-induced serpin secreted by the porcine uterus, and the expression of its mRNA during pregnancy. Molecular Endocrinology. 4(3):428-40, 1990

Mangelsdorf DJ. Ong ES. Dyck JA. Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature. 345(6272):224-9, 1990

Mathialagan N. Bixby JA. Roberts RM. Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. Molecular Reproduction & Development. 32(4):324-30, 1992

Matrisian LM . The matrix-degrading metalloproteinases. Bioessays. 14(7):455-63, 1992

Mattei MG. de The H. Mattei JF. Marchio A. Tiollais P. Dejean A. Assignment of the human hap retinoic acid receptor RAR beta gene to the p24 band of chromosome 3. Human Genetics. 80(2):189-90, 1988b

Mattei MG. Petkovich M. Mattei JF. Brand N. Chambon P. Mapping of the human retinoic acid receptor to theq21 band of chromosome 17. Human Genetics. 80(2):186-8, 1988a

Mattson BA. Overstrom EW. Albertini DF. Transitions in trophectoderm cellular shape and cytoskeletal organization in the elongating pig blastocyst. Biology of Reproduction. 42(1):195-205, 1990

McLaren A. The embryo. In Embryonic and Fetal Development, ed. by C.R. Austin & R.V. Short. Cambridge University Press, UK, 1972.

Menino AR Jr. Hogan A. Schultz GA. Novak S. Dixon W. Foxcroft GR. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. Developmental Genetics. 21(1):68-74, 1997

Morgan GL. Geisert RD. Zavy MT. Shawley RV. Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. Journal of Reproduction & Fertility. 80(1):125-31, 1987a

Morgan GL. Geisert RD. Zavy MT. Fazleabas AT. Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. Journal of Reproduction & Fertility. 80(1):133-41, 1987b

Mullins DE. Bazer FW. Roberts RM. Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell. 20(3):865-72, 1980

Murray MK. Malathy PV. Bazer FW. Roberts RM. Structural relationship, biosynthesis, and immunocytochemical localization of uteroferrin-associated basic glycoproteins. Journal of Biological Chemistry. 264(7):4143-50, 1989

Nie GY. Butt AR. Salamonsen LA. Findlay JK. Hormonal and non-hormonal agents at implantation as targets for contraception. Reproduction, Fertility, & Development. 9(1):65-76, 1997

Nishida T. Murakami J. Otori T. Expression of fibronectin receptor (integrin) in the uterus of rats in relation to the estrous cycle. Histochemistry. 96(4):279-83, 1991

Niswender GD and Nett TM. In The Physiology of Reproduction, ed. by E. Knobil and J. Neill, Raven, New York, pp. 489-525, 1988

Nuttleman PR. Roberts RM. Transfer of iron from uteroferrin (purple acid phosphatase) to transferrin related to acid phosphatase activity. Journal of Biological Chemistry. 265(21):12192-9, 1990

Papaioannou VE. Ebert KM. The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. Development. 102(4):793-803, 1988

Parsons JT. Parsons SJ. Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Current Opinion in Cell Biology. 9(2):187-92, 1997

Parsons SL. Watson SA. Brown PD. Collins HM. Steele RJ. Matrix metalloproteinases. British Journal of Surgery. 84(2):160-6, 1997

Patten BM. Embryology of the Pig (3rd edition). McGraw-Hill Book Company, New York, USA. 1948.

Pei D. Identification and characterization of the fifth membrane-type matrix metalloproteinase MT5-MMP. Journal of Biological Chemistry. 274(13):8925-32, 1999

Perry JS. Heap RB. Amoroso EC. Steroid hormone production by pig blastocysts. Nature. 245(5419):45-7, 1973

Perry JS. Heap RB. Burton RD. Gadsby JE. Endocrinology of the blastocyst and its role in the establishment of pregnancy. Journal of Reproduction & Fertility - Supplement. (25):85-104, 1976

Perry JS. Rowlands IW. Early pregnancy in the pig. Journal of Reproduction and Fertility. 4:175-88, 1962.

Perry JS. The mammalian fetal membranes. Journal of Reproduction & Fertility. 62(2):321-35, 1981

Polan ML. Simon C. Frances A. Lee BY. Prichard LE . Role of embryonic factors in human implantation. Human Reproduction. 10 Suppl 2:22-9, 1995

Pollard JW. Hunt JS. Wiktor-Jedrzejczak W. Stanley ER . A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Developmental Biology. 148(1):273-83, 1991

Pope WF, Xie S, Broermann DM. Nephew KP. Causes and consequences of early embryonic diversity in pigs. Journal of Reproduction and Fertility Supplement, 40: 251-260, 1990

Pope WF. Embryonic mortality in swine. In Embryonic Mortality in Domestic Species, ed. by Michael T. Zavy and Rodney D. Geisert, CRC Press, Boca Raton, Florida, USA. Pg. 53-77, 1994

Pope WF. First NL. Factors affecting the survival of pig embryos. Theriogenology. 23:91-105, 1985.

Pope WF. Maurer RR. Stormshak F. Intrauterine migration of the porcine embryo--interaction of embryo, uterine flushings and indomethacin on myometrial function in vitro. Journal of Animal Science. 55(5):1169-78, 1982

Puente XS. Pendas AM. Llano E. Velasco G. Lopez-Otin C. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. Cancer Research. 56(5):944-9, 1996

Pusateri AE. Rothschild MF. Warner CM. Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. Journal of Animal Science. 68(11):3727-35, 1990

Ramsoondar J, Christopherson RJ, Guilbert LJ, Wegmann T (1993): A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biol. Reprod. 49: 681-694.

Ramsoondar J. (1994) The immunobiology of the porcine placenta (Ph.D. thesis). University of Alberta, Edmonton, Canada. pp. 226.

Reponen P. Leivo I. Sahlberg C. Apte SS. Olsen BR. Thesleff I. Tryggvason K. 92-kDa type IV collagenase and TIMP-3, but not 72-kDa type IV collagenase or TIMP-1 or TIMP-2, are highly expressed during mouse embryo implantation. Developmental Dynamics. 202(4):388-96, 1995

Rivera RM. Youngs CR. Ford SP. A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages. Journal of Reproduction & Fertility. 106(1):111-6, 1996

Roberts AB. Sporn MB. Transforming growth factor beta. Advances in Cancer Research. 51:107-45, 1988

Roberts RM. Xie S. Trout WE. Embryo-uterine interactions in pigs during week 2 of pregnancy. Journal of Reproduction & Fertility - Supplement. 48:171-86, 1993

Roberts RM. Cross JC. Farin CE. Hansen TR. Klemann SW. Imakawa K. Interferons at the placental interface. Journal of Reproduction & Fertility - Supplement. 41:63-74, 1990

Roberts RM. Bazer FW. The functions of uterine secretions. Journal of Reproduction & Fertility. 82(2):875-92, 1988

Roberts RM. Raub TJ. Bazer FW. Role of uteroferrin in transplacental iron transport in the pig. Federation Proceedings. 45(10):2513-8, 1986

Ross AC. Vitamin A: current understanding of the mechanisms of action. Nutrition Today. 1:6-12, 1991.

Ross SA. Ahrens RA. De Luca LM. Retinoic acid enhances adhesiveness, laminin and integrin beta 1 synthesis, and retinoic acid receptor expression in F9 teratocarcinoma cells. Journal of Cellular Physiology. 159(2):263-73, 1994

Ryu OH. Fincham AG. Hu CC. Zhang C. Qian Q. Bartlett JD. Simmer JP. Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. Journal of Dental Research. 78(3):743-50, 1999

Salamonsen LA. Role of proteases in implantation. Reviews of Reproduction. 4(1):11-22, 1999

Samuel CA. Perry JS. The ultrastructure of pig trophoblast rransplanted to an ectopic site in the uterine wall. Journal of Anatomy. 113(1):139-49, 1972a

Samuel CA. The development of pig trophoblast in ectopic sites. Journal of Reproduction & Fertility. 27(3):494-5, 1971

Samuel CA. Ultra-structural features of the ectopic pig trophoblast. Journal of Physiology. 226(2):22P, 1972b

Sapi E. Flick MB. Rodov S. Gilmore-Hebert M. Kelley M. Rockwell S. Kacinski BM . Independent regulation of invasion and anchorage-independent growth by different autophosphorylation sites of the macrophage colony-stimulating factor 1 receptor. Cancer Research. 56(24):5704-12, 1996

Sato H. Okada Y. Seiki M. Membrane-type matrix metalloproteinases (MT-MMPs) in cell invasion. Thrombosis & Haemostasis. 78(1):497-500, 1997

Sawai K. Matsuzaki N. Okada T. Shimoya K. Koyama M. Azuma C. Saji F. Murata Y. Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones. Biology of Reproduction. 56(5):1274-80, 1997

Schlaepfer DD. Hanks SK. Hunter T. van der Geer P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature. 372(6508):786-91, 1994

Schule R. Muller M. Kaltschmidt C. Renkawitz R. Many transcription factors interact synergistically with steroid receptors. Science. 242(4884):1418-20, 1988a

Schule R. Muller M. Otsuka-Murakami H. Renkawitz R. Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature. 332(6159):87-90, 1988b

Schultz GA. Edwards DR. Biology and genetics of implantation. Developmental Genetics. 21(1):1-5, 1997

Schultz GA. Heyner S. Growth factors in preimplantation mammalian embryos. Reviews of Reproductive Biology. 15:43-81, 1993

Scofield AM. Clegg FG. Lamming GE. Embryonic mortality and uterine infection in the pig. Journal of Reproduction & Fertility. 36(2):353-61, 1974

Sehgal I. Thompson TC. Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-beta1 in human prostate cancer cell lines. Molecular Biology of the Cell. 10(2):407-16, 1999

Shaw AR. Domanska A. Mak A. Gilchrist A. Dobler K. Visser L. Poppema S. Fliegel L. Letarte M. Willett BJ. Ectopic expression of human and feline CD9 in a human B cell line confers beta 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. Journal of Biological Chemistry. 270(41):24092-9, 1995

Shellard J. Perreau J. Brulet P. Role of leukemia inhibitory factor during mammalian development. European Cytokine Network. 7(4):699-712, 1996

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

Shimonovitz S. Hurwitz A. Dushnik M. Anteby E. Geva-Eldar T. Yagel S. Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. American Journal of Obstetrics & Gynecology. 171(3):832-8, 1994

Shuai K. Interferon-activated signal transduction to the nucleus. Current Opinion in Cell Biology. 6(2):253-9, 1994

Simmen FA. Simmen RC. Geisert RD. Martinat-Botte F. Bazer FW. Terqui M. Differential expression, during the estrous cycle and pre- and postimplantation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system. Endocrinology. 130(3):1547-56, 1992

Simmen FA. Simmen RC. Peptide growth factors and proto-oncogenes in mammalian conceptus development. Biology of Reproduction. 44(1):1-5, 1991

Simmen RC. Baumbach GA. Roberts RM. Molecular cloning and temporal expression during pregnancy of the messenger ribonucleic acid encoding uteroferrin, a progesterone-induced uterine secretory protein. Molecular Endocrinology. 2(3):253-62, 1988

Simon C. Moreno C. Remohi J. Pellicer A. Cytokines and embryo implantation. Journal of Reproductive Immunology. 39(1-2):117-31, 1998

Simon C. Frances A. Piquette G. Hendrickson M. Milki A. Polan ML. Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. Journal of Clinical Endocrinology & Metabolism. 78(4):847-54, 1994a

Simon C. Frances A. Piquette GN. el Danasouri I. Zurawski G. Dang W. Polan ML. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology. 134(2):521-8, 1994b

Simon C. Mercader A. Gimeno MJ. Pellicer A. The interleukin-1 system and human implantation. American Journal of Reproductive Immunology. 37(1):64-72, 1997

Simon C. Pellicer A. Polan ML . Interleukin-1 system crosstalk between embryo and endometrium in implantation. Human Reproduction. 10 Suppl 2:43-54, 1995

Simon C. Piquette GN. Frances A. Polan ML . Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. Journal of Clinical Endocrinology & Metabolism. 77(2):549-55, 1993

Slack J. McMahan CJ. Waugh S. Schooley K. Spriggs MK. Sims JE. Dower SK . Independent binding of interleukin-1 alpha and interleukin-1 beta to type I and type II interleukin-1 receptors. Journal of Biological Chemistry. 268(4):2513-24, 1993

Smith AG. Heath JK. Donaldson DD. Wong GG. Moreau J. Stahl M. Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature. 336(6200):688-90, 1988

Smith DF. Toft DO. Steroid receptors and their associated proteins. Molecular Endocrinology. 7(1):4-11, 1993

Sporn MB. Roberts AB. Roche NS. Kagechika H. Shudo K. Mechanism of action of retinoids. Journal of the American Academy of Dermatology. 15(4):756-64, 1986

Stallings-Mann M. Trout WE. Roberts RM. A novel family of progesterone-induced, retinol-binding proteins from uterine secretions of the pig. Journal of Biological Chemistry. 266(34):23515, 1991

Stallings-Mann ML. Burke MG. Trout WE. Roberts RM. Purification, characterization, and cDNA cloning of a Kunitz-type proteinase inhibitor secreted by the porcine uterus. Journal of Biological Chemistry. 269(39):24090-4, 1994

Stallings-Mann ML. Trout WE. Roberts RM. Porcine uterine retinol-binding proteins are identical gene products to the serum retinol-binding protein. Biology of Reproduction. 48(5):998-1005, 1993

Stanley ER. Guilbert LJ. Tushinski RJ. Bartelmez SH . CSF-1--a mononuclear phagocyte lineage-specific hemopoietic growth factor. Journal of Cellular Biochemistry. 21(2):151-9, 1983

Stewart CL . The role of leukemia inhibitory factor (LIF) and other cytokines in regulating implantation in mammals. Annals of the New York Academy of Sciences. 734:157-65, 1994

Stewart CL. Cullinan EB. Preimplantation development of the mammalian embryo and its regulation by growth factors. Developmental Genetics. 21(1):91-101, 1997

Stewart CL. Kaspar P. Brunet LJ. Bhatt H. Gadi I. Kontgen F. Abbondanzo SJ . Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature. 359(6390):76-9, 1992

Stolow MA. Bauzon DD. Li J. Sedgwick T. Liang VC. Sang QA. Shi YB. Identification and characterization of a novel collagenase in Xenopus laevis: possible roles during frog development. Molecular Biology of the Cell. 7(10):1471-83, 1996

Stone BA. Seamark RF. Kelly RW. Deam S. Production of steroids and release of prostaglandins by spherical pig blastocysts in vitro. Australian Journal of Biological Sciences. 39(3):283-93, 1986

Stroband HW. Van der Lende T. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction & Fertility - Supplement. 40:261-77, 1990

Stroband HW. Van der Lende T. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction & Fertility - Supplement. 40:261-77, 1990

Surveyor GA. Gendler SJ. Pemberton L. Das SK. Chakraborty I. Julian J. Pimental RA. Wegner CC. Dey SK. Carson DD. Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology. 136(8):3639-47, 1995

Szafranska B. Xie S. Green J. Roberts RM. Porcine pregnancy-associated glycoproteins: new members of the aspartic proteinase gene family expressed in trophectoderm. Biology of Reproduction. 53(1):21-8, 1995

Tabibzadeh S. Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. Human Reproduction. 7(6):876-82, 1992

Takacs L. Kovacs EJ. Smith MR. Young HA. Durum SK . Detection of IL-1 alpha and IL-1 beta gene expression by in situ hybridization. Tissue localization of IL-1 mRNA in the normal C57BL/6 mouse. Journal of Immunology. 141(9):3081-95, 1988

Thatcher WW. Hansen PJ. Gross TS. Helmer SD. Plante C. Bazer FW. Antiluteolytic effects of bovine trophoblast protein-1. Journal of Reproduction & Fertility - Supplement. 37:91-9, 1989

Thompson JN. Howell J. Pitt GAJ. Vitamin A and reproduction in rats. Proceedings of Royal Society London Series 159B, 510-535, 1993.

Tienari J. Alanko T. Lehtonen E. Saksela O. The expression and localization of urokinase-type plasminogen activator and its type 1 inhibitor are regulated by retinoic acid and fibroblast growth factor in human teratocarcinoma cells. Cell Regulation. 2(4):285-97, 1991

Trout WE. Hall JA. Stallings-Mann ML. Galvin JM. Anthony RV. Roberts RM. Steroid regulation of the synthesis and secretion of retinol-binding protein by the uterus of the pig. Endocrinology. 130(5):2557-64, 1992

Trout WE. McDonnell JJ. Kramer KK. Baumbach GA. Roberts RM. The retinol-binding protein of the expanding pig blastocyst: molecular cloning and expression in trophectoderm and embryonic disc. Molecular Endocrinology. 5(10):1533-40, 1991

Tuo W. Harney JP. Bazer FW. Developmentally regulated expression of interleukin-1 beta by peri-implantation conceptuses in swine. Journal of Reproductive Immunology. 31(3):185-98, 1996

Tuo W. Harney JP. Bazer FW. Colony-stimulating factor-1 in conceptus and uterine tissues in pigs. Biology of Reproduction. 53(1):133-42, 1995

Van Der Lende T. Soede NM. Kemp BV. Embryo mortality and prolificacy in the pig. In Principles of Pig Science, ed. by Cole DJA. Wiseman J. Varley MA, Nottingham University Press, pp. 297-317, 1994.

Vaughan TJ. James PS. Pascall JC. Brown KD. Expression of the genes for TGF alpha, EGF and the EGF receptor during early pig development. Development. 116(3):663-9, 1992

Veerapandian B. Structure and function of interleukin-1, based on crystallographic and modeling studies. Biophysical Journal. 62(1):112-5, 1992

Waterhouse P. Denhardt DT. Khokha R . Temporal expression of tissue inhibitors of metalloproteinases in mouse reproductive tissues during gestation. Molecular Reproduction & Development. 35(3):219-26, 1993

Wegner CC. Carson DD. Cell adhesion processes in implantation. Oxford Reviews of Reproductive Biology. 16:87-137, 1994.

Weigel NL. Steroid hormone receptors and their regulation by phosphorylation. Biochemical Journal. 319 (3):657-67, 1996

Weitlauf, HM. Biology of implantation, in The Physiology of Reproduction, ed. by E. Knobil and J. Neil et al., Raven Press, New York. 1988. Pp. 231-267

Wildt DE. Riegle GD. Dukelow WR. Physiological temperature response and embryonic mortality in stressed swine. American Journal of Physiology. 229(6):1471-5, 1975

Williams BRG. Haque SJ. Interacting pathways of interferon signaling. Seminars in Oncology. 24 (3) Supplement 9:70-77, 1997.

Wilson ME. Ford SP. Differences in trophectoderm mitotic rate and P450 17alpha-hydroxylase expression between late preimplantation Meishan and Yorkshire conceptuses. Biology of Reproduction. 56(2):380-5, 1997

Wong GG. Temple PA. Leary AC. Witek-Giannotti JS. Yang YC. Ciarletta AB. Chung M. Murtha P. Kriz R. Kaufman RJ. et al. Human CSF-1: molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. Science. 235(4795):1504-8, 1987

Wrathall AE. Prenatal survival in pigs. Part 1, Ovulation rate and its influence on prenatal survival and litter size in pigs, Rev. Series No. 9, Commonwealth Bureau of Animal Health, Farnham Royal, Slough, England, 1971.

Yagel S. Lala PK. Powell WA. Casper RF . Interleukin-1 stimulates human chorionic gonadotropin secretion by first trimester human trophoblast. Journal of Clinical Endocrinology & Metabolism. 68(5):992-5, 1989

Yelich JV. Pomp D. Geisert RD. Detection of transcripts for retinoic acid receptors, retinol-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. Biology of Reproduction. 57(2):286-94, 1997b

Yelich JV. Pomp D. Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biology of Reproduction 57:1256-65, 1997

Yoo HJ. Barlow DH. Mardon HJ. Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. Developmental Genetics. 21(1):102-8, 1997

Yu VC. Delsert C. Andersen B. Holloway JM. Devary OV. Naar AM. Kim SY. Boutin JM. Glass CK. Rosenfeld MG. RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell. 67(6):1251-66, 1991

Yurochko AD. Adhesion molecules as signal transduction molecules. In Adhesion molecules in Health and Disease, ed. by Paul LC. Issekutz TB., Marcel Dekker, Inc., New York, 1997.

CHAPTER 2

CSF-1/C-FMS IS AN IMPORTANT AUTOCRINE AND PARACRINE GROWTH FACTOR-RECEPTOR PATHWAY FOR REGULATING PROLIFERATION OF PORCINE TROPHOBLAST CELLS

INTRODUCTION

Macrophage colony-stimulating factor (CSF-1) is a glycoprotein which was initially identified as a hematopoietic growth factor involved in the proliferation and differentiation of monocytic cells in both the human and the mouse (reviewed in Stanley et al., 1983 and Stanley, 1990). CSF-1 has since been shown to be involved in a host of diverse biological activities, mediated through its cell surface tyrosine kinase receptor (CSF-1R) (the product of the *c-fms* protooncogene) (Sherr et al., 1985). These include the regulation of early embryonic and placental development (Pollard, 1987 and 1990; Pampfer et al., 1991), uterine growth and function prior to and during gestation (Pampfer et al., 1991; Bartocci et al., 1986; Arceci et al., 1989; Pollard, 1991; Daiter et al., 1992), as well as a role in the pathogenesis of human carcinomas (Filderman et al., 1992). More recently, studies on CSF-1 nullizygous mice ($csfm^{op}/csfm^{op}$) have revealed other important functions of CSF-1 in gametogenesis, gonadal steroidogenesis and mammary gland development during pregnancy (Sapi and Kacinski, 1999; Cohen et al., 1996 & 1997; Pollard 1997). In addition, the expression of CSF-1 and its receptor has been positively correlated with the invasiveness of tumors of the human breast, ovary, and endometrium (Sapi et al., 1996; Kacinski, 1997).

In both the human and the mouse, the CSF-1 gene is present as a single-copy in the genome (Kawasaki et al., 1985; Ladner et al., 1987; Rajavashisth et al., 1987). The human *csf-1* consists of 10 exons and 9 introns, which are present on a genomic DNA fragment of about 21 kb situated on the long arm of chromosome 5 (Kawasaki and

Ladner, 1990; Pettenati et al., 1987). The human CSF-1 gene is expressed in many types of tissues and cells and in many cases transcript size heterogeneity has been observed. Of at least seven species of mRNA detected in human cells, five cDNAs corresponding to the 1.6-, 2.2-, 2.5-, 3.0-, and 4.0-kb mRNA species have been cloned (Kawasaki et al., 1985; Wong et al., 1987; Ladner et al., 1987; Cerreti et al., 1988; Pampfer et al., 1991). Three isoforms of a CSF-1 protein precursor could be deduced from the five cDNA sequences characterized, which are CSF-1^{554} [554 aa in length], CSF-1^{438}, and CSF-1^{256} (Fixe and Praloran, 1997; Deng et al., 1996). The first two precursors are ultimately secreted as soluble growth factors, while the shortest precursor is membrane-bound and externalized at the cell surface, where it is slowly released by extracellular proteolysis (Rettenmier et al., 1987; Pampfer et al., 1991). In the mouse, cDNAs of two major mRNA species (2.3 and 4.0 kb) of mouse CSF-1 have been cloned (Ladner et al., 1988). The coding sequence of rat CSF-1 cDNA has been shown to be identical to that of mouse CSF-1 (Borycki et al., 1993).

The human c-fms proto-oncogene is about 75 kb in length, consisting of 22 exons and 21 introns, which is also located on the long arm of chromosome 5 (Sherr, 1990; Groffen et al., 1983). Exon 1 is transcribed in placental cells but not in macrophages due to the differential transcription of the c-fms gene by two different tissue-specific promoters, and multiple initiation sites for transcription appear to be utilized in placental cells (Visvader and Verma, 1989). Expression of CSF-1R mRNA, but not CSF-1 mRNA, was detected at low levels in mouse embryos throughout pre-implantation development (Arceci et al., 1992).

Using a human CSF-1 cDNA and an anti-human CSF-1 monoclonal antibody, the expression of CSF-1 at both the mRNA and protein level were demonstrated in porcine uterine, conceptus, allantochorion and fetal tissues during post-implantation stages of pregnancy (Tuo et al., 1995). However, to date, there has been no published information on the sequence of porcine CSF-1 cDNA. In this study, we provide evidence that CSF-1/c-fms is an important autocrine/paracrine growth factor-receptor pathway for regulating proliferation of porcine trophoblast cells. In addition, we describe for the first time the cDNA sequence encoding porcine CSF-1, demonstrate that the CSF-1 gene is present as a single copy in the porcine genome, and examine the mRNA expression of CSF-1 and c-fms in endometrial and embryonic tissues during pre-implantation development.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight and having two recorded estrous cycles were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of standing heat with a vasectomized boar. At 16 and 24 hours after onset of standing heat (Day 0 of pregnancy), gilts were artificially inseminated using fresh, pooled, crossbred semen. Pregnant gilts were slaughtered on different days of pregnancy, and the reproductive tract was recovered within 10 minutes of death.

B. Collection of Endometrial and Embryonic Tissues

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed once in a saline buffer. Spherical blastocsyts were sorted according to their size and

grouped as S1 (1-2 mm in diameter), S2 (5-6 mm) and S3 (8-10 mm). Elongated blastocysts, including ovoid, tubular and filamental stages, were pooled for individual pigs at different stages of pregnancy since they were frequently clumped together in the flushings. At the same time, sections of endometrial tissues were also harvested from the mid-section of each of two uterine horns for each individual pig. All the samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. Cell Culture

A trophoblast cell line, Jag-1, was originally isolated from trophoblastic tips of Day 14 porcine embryos, and was maintained as described (Ramsoondar et al., 1993). Porcine macrophages were isolated from "buffy coats" prepared from whole pig blood (Ramsoondar et al., 1993). Both porcine macrophages and Jag-1 cells were cultured in RPMI containing 10% (v/v) bovine calf serum and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Two human small-cell lung cancer cell lines (H69B and H69C) (Dixon et al., 1995; Feyles et al., 1991) were included as negative controls for examination of CSF-1 expression, and were maintained in RPMI medium supplemented with 6% (v/v) bovine calf serum.

D. Cell Proliferation Bioassay

Jag-1 cells were cultured in SFM for 72 hr, and the conditioned medium was collected and concentrated about 10 times by filtration through a YM 10 (10k MW cut-off) membrane (Amicon, USA) within an ultrafiltration cell at 4 °C.

Proliferation bioassays of porcine macrophages and Jag-1 cells were carried out basically as described by Ramsoondar et al. (1993). Briefly, cells (5 x 10^4 cells/ well) in 100 µl of serum-free medium (SFM) (Gibco/BRL) were plated in each well of flat-

bottomed 96-well plates and were cultured for 2 hr. The medium in each well was replaced with 100 µl of test media and the cells were allowed to proliferate for a further 48 hr. The cells were subsequently pulsed by adding tritiated thymidine (1 µCi/well) in 20 µl SFM and further incubated for 18 hr. The cells were finally harvested and counted for tritiated thymidine incorporation using a Matrix 96 counter (Canberra Packard, USA).

A YY106 hybridoma cell line, producing anti-human CSF-1 monoclonal antibody, was kindly provided by Dr. L. Guilbert (University of Alberta).

E. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL). TRIzol reagent was directly added to the frozen embryonic tissues or cultured cells, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen prior to addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by spectrophotometry.

F. Primer Design and RT-PCR

The coding sequences of human and mouse CSF-1 and c-fms were retrieved from the GenBank database (NCBI) and aligned and compared with the aid of a computer program, GeneJockey II (Biosoft, Cambridge, UK). Highly conserved regions between the two species, which are found at both ends of the CSF-1 coding sequence, were used to design primers for amplifying the cDNA sequence of porcine CSF-1 (Wong et al., 1987 and Ladner et al., 1988). The consensus sequences at the 3'-end of human and mouse c-fms coding sequences were used to generate primers for amplification of porcine

c-fms cDNA fragments (Rothwell and Rohrschneider, 1987; Coussens et al., 1986). DNA fragments from glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and/or β-actin cDNA sequences were amplified as internal controls for gene expression using primers described previously (Yelich et al., 1997; Table 2.1). All primers and their sequences are shown in Table 2.1.

For amplification with the primer pair CSF-1U and CSF-1D, 1 μg of total RNA and 50 picomoles of the downstream primer (CSF-1D) were used for the reverse transcription (RT) reaction in a volume of 20 μl containing 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, 0.5% Tween 20 (v/v), 1 mM dNTP, 10 mM DTT and 50 units Expand™ reverse transcriptase (Boehringer Mannheim). The RT reaction was carried out at 42°C for 1 hr, followed by incubation at 95 °C for 5 min. The RT product of 4 μl was then used directly for PCR in a 50-μl reaction volume containing 50 mM Tris-HCl pH 9.2, 160 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 350 μM dNTP, 300 nM each of CSF-1U and CSF-1D primers, and 2.5 units Expand™ long-template polymerase (Boehringer Mannheim). PCR parameters were denaturation at 94°C for 2 min, 30 cycles of (94 °C 10 sec, 65 °C 30 sec and 68 °C 2 min) and an additional 7-min extension at 68 °C.

For amplification of DNA fragments less than 1 kb in size, RT was basically carried out as described above, except oligo$(dT)_{15}$ primer was used instead of CSF-1D. PCR was carried out in a 50-μl reaction volume containing 4μl of RT products (only 0.1 μl for G3PDH and β-actin), 20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 200 nM each of upstream and downstream primers and 1.25 units *Taq* polymerase (Gibco/BRL).

G. Northern and Southern Blot Analysis

Probe DNA for both Northern and Southern blot hybridization was prepared using the DNA Random Primers Labeling Kit (Gibco/BRL) following the manufacturer's recommendations. Northern blots were prepared on nitrocellulose membranes (Micron Separations Inc., Massachusetts, USA). Total RNA was fractionated on a 1% (w/v) agarose gel containing 7% (v/v) formaldehyde in MOPS buffer. Northern hybridization was carried out at 65°C for 18 hr in 6x SSPE buffer containing 0.5% SDS and 5x Denhardt's solution. The blot was washed 3x 15 min in 2x SSC [0.1% (w/v) SDS] at room temperature and 1x 15 min in 0.1x SSPE [0.1% (w/v) SDS], and it was exposed to a Kodak XAR film for 48 hr at -80 °C.

Genomic DNA was isolated from Jag-1 cells as described (Ausubel et al., 1996), and digested with either *Bam* HI, *Eco* RI, *Hind* III or *Pst* I restriction endonucleases, respectively. Digests of 20 µg of DNA for each enzyme were fractionated on 0.8% (w/v) agarose gel in 1xTAE buffer at 2 V/cm for 10 hr. DNA capillary transfer was carried out in 0.4 M NaOH using Zeta-Probe membranes (Bio-Rad). Southern hybridization was carried out following the manufacturer's recommendations. Briefly, hybridization was carried out overnight at 43 °C in a solution containing 50% (v/v) formamide, 0.12 M Na_2HPO_4, 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA, pH7.2. The blot was washed once 15 min at room temperature in both 2x SSC [0.1% (w/v) SDS] and 0.5x SSC [0.1% (w/v) SDS], respectively, and then once at 65 °C in 0.1x SSC [0.1% (w/v) SDS]. Exposure to a Kodak XAR film was done at -80 °C for 7 days.

RESULTS

A. Cell Proliferation Bioassay

The conditioned medium of Jag-1 cells was shown to stimulate the proliferation of porcine macrophages in a dose dependent fashion (Fig. 2.1A). This medium, when fractionated by reverse phase–FPLC, gave a single peak of activity with respect to the macrophage bioassay (data not shown). CSF-1 has been shown to be potent growth factor involved in the proliferation and differentiation of monocytic cells in both the human and the mouse (Stanley et al., 1983) and is therefore a potential candidate for the unknown porcine macrophage growth factor. Using a 1:1 (50%) dilution of the Jag-1 conditioned medium, an anti-human CSF-1 neutralizing monoclonal antibody YY106 was able to inhibit in a dose-dependent fashion the stimulatory activity in Jag-1 cell-conditioned medium (Fig. 2.1A). In addition, it was found in a parallel *in vitro* proliferation assay that YY106 could inhibit the proliferation of Jag-1 cells in a dose-dependent manner (Fig. 2.1B).

B. Cloning and Sequencing of Two Overlapping Fragments of a Porcine CSF-1 cDNA sequence

Using a RT-PCR approach, one predicted cDNA fragment of about 1.5 kb was amplified from Jag-1 total cellular RNA using the primer pair CSF-1U & CSF-1D (Fig. 2.2A), whereas no predicted fragment was amplified with another primer pair CSF-1S & CSF-1D (data not shown). The yield of the 1.5-kb amplicon from first round RT-PCR was low as indicated in Figure 2.2A and was insufficient for further analysis. Therefore, it was excised from the agarose gel using a clean razor blade and the gel slice was soaked

in 100 µl of distilled H_2O overnight at 4 °C. Sufficient yield was obtained after re-amplification with 5 µl of the elate using the same PCR conditions (Fig. 2.2B).

The 1.5-kb amplicon was cloned into the pCR-script Amp SK (+) vector (Stratagene) and was sequenced in both directions on an Automatic Sequencer (ABI/Prism 2.2.1). The resulting sequence shared significant homologies with human and mouse CSF-1 cDNA sequences. In order to obtain the missing 5'-end of the coding sequence for porcine CSF-1 gene, an internal primer, CSF-13 (Table 2.1 and Fig. 2.6), was designed according to the 5'-end sequence of the 1,569-nt cDNA fragment of porcine CSF-1 obtained above. RT was performed with the primer CSF-1D (Table 2.1 and Fig. 2.5). *Taq* polymerase (Gibco/BRL) was used for the subsequent PCR reaction with the primers CSF-1S and CSF-13 instead of Expand™ Long Template Polymerase used previously, because the predicted size of the PCR product from primers CSF-1S and CSF-13 was small. An amplicon with the expected size of 203 bp was obtained after RT-PCR. The identity of this amplicon was further confirmed by diagnostic restriction digestion with Pst I (Fig. 2.4) that has a restriction site at the 5'end of the 1,569-bp amplicon. The 203-bp amplicon was cloned and sequenced as described above. Therefore, the full-length coding sequence (1, 665 nt in length) of a porcine CSF-1 cDNA was obtained after alignment between the 1,569 nt and 203 nt overlapping partial sequences.

C. The Coding Sequence and Deduced Amino Acid Sequence of Porcine CSF-1

The full coding sequence of porcine CSF-1 was found to be 1,665 nt in length by assembling the sequences of the two overlapping amplicons of 1,569 bp and 203 bp, and encodes a protein precursor of 554aa with a 32aa peptide signal sequence (Fig. 2.5).

The coding sequence of porcine CSF-1 shares 82 % and 73 % sequence identity with human CSF-1 (Wong et al., 1987) and mouse CSF-1 (Ladner et al., 1988), respectively. The deduced amino acid sequence of porcine CSF-1 shares 73 % and 65 % homology with those of the human CSF-1 and the mouse CSF-1, respectively (Table 2.2). In addition, most of the structural features found in the predicted porcine CSF-1 precursor are common to those seen in the human and mouse proteins. Thirteen cysteines exist in the porcine CSF-1 precursor, twelve of which are conserved in the human and mouse proteins (Fig. 2.6). With the exception of the site at position N38, the other two sites of potential N-linked glycosylation at positions of N154 and N172 in porcine CSF-1 are conserved in human and mouse CSF-1 (Table 2.2). The glycosaminoglycan addition site is present as a short sequence "EEASGEASE" and is identical among the three CSF-1 precursors (Fig. 2.6). A highly hydrophobic domain from F495 to Y517 is followed by a short stretch of positively charged residues (R-R-R-R-W) and a cytoplasmic tail in the porcine CSF-1, which is typical of a transmembrane domain similar to those found in the human and mouse CSF-1 proteins (Fig. 2.6).

D. Expression of CSF-1 in Jag-1 Cells and Southern Blot Analysis of Porcine CSF-1 Gene

The cDNA fragment of 1,569 bp was used as a probe to examine the expression of the CSF-1 gene in cells of Jag-1, H69B and H69C cultured cells. Under the stringent washing conditions used (0.1x SSPE at 68°C), only one species of mRNA (about 4.0 kb) was detected in Jag-1 cells, and no hybridization signal was observed in either H69B or H69C human carcinoma cells, even after long exposure times (Fig. 2.3).

It was of interest to determine the number of copies of the CSF-1 gene in the porcine genome, since this information is needed for further studies on the structure and regulation of expression of the porcine CSF-1 gene. For this purpose, genomic DNA was extracted from Jag-1 cells and digested with four different restriction enzymes. Southern blot analysis was carried out using a radiolabeled porcine cDNA fragment of 1,569 bp as probe. One hybridizing fragment with approximate sizes of 2.2 kb, 23.0 kb and 25.0 kb was found in the *Bam* HI, *Eco* RI and *Hind* III digests, respectively, and two hybridizing fragments of about 1.3 kb and 4.6 kb in the *Pst* I digests (Fig. 2.7). These results suggest that there is only one copy of the CSF-1 gene in the pig genome.

E. Expression of CSF-1 transcripts in Endometrial and Embryonic Tissues during Pre-implantation Development

A major CSF-1 mRNA species of approximately 4 kb was detected by Northern blot analysis in RNA extracted from endometrial tissues at all pre-implantation developmental stages examined and in Jag-1 cells. Two minor mRNA species of about 5.1 kb and 3.6 kb, respectively, were also detected in endometrial tissues around Day 16 of pregnancy but not in Jag-1 cells (Fig. 2.3 and 2.8). Expression levels of the 4-kb CSF-1 mRNA in endometrial tissues of each of the pre-implantation developmental stages were normalized as a percentage of the 28S ribosomal RNA expression at their corresponding stages. High levels of CSF-1 mRNA expression were found in endometrial tissues at Day 3 of pregnancy, followed by a basal level of expression between Days 4 and 15.5 of pregnancy (Fig. 2.9). By Day 16 of pregnancy, CSF-1 mRNA level is elevated about 3 fold over basal levels in endometrial tissues.

CSF-1 mRNA levels in endometrial and embryonic tissues were compared at six different stages between Days 11 and 16 of pregnancy using RT-PCR with primers CSF-1S and CSF-13, since total RNA amounts obtained from embryos at earlier stages were limited. CSF-1 mRNA expression was up-regulated in embryonic tissues at the same period of time as in endometrial tissues with highest levels seen at Day 16. However, the absolute expression levels in embryonic tissues were much lower than in endometrial tissues at the six stages examined (Fig. 2.10).

F. Expression of c-fms transcripts in Embryonic and Endometrial Tissues during the Peri-implantation Period

A pair of primers, PR3 and PR6, was designed based on the 3'-end consensus regions of human and mouse c-fms coding sequences. A 303-bp cDNA fragment of porcine c-fms was amplified, cloned and sequenced from endometrial tissue RNA using the RT-PCR approach (Fig. 2.11). This fragment showed 85% and 79% sequence identity with the correspondent regions of human and mouse c-fms cDNA sequences. The deduced amino acid sequences shared 86% and 81% sequence identity with human and mouse sequences. Expression of c-fms mRNA was detected in endometrial tissues obtained between Days 9 and 15.75 of pregnancy using RT-PCR, but surprisingly not in blastocysts (Fig. 2.12A&D). Since we had already obtained strong evidence of a functionally signal pathway involving CSF-1 and, by definition, its receptor in embryo-derived trophoblast cells (Fig. 1), it was important to reconcile this discrepancy. Therefore, a pair of nested-primers CSF-1R7 and CSF-1R8, located within the 303-nt c-fms cDNA sequence characterized above, was needed to carry out a second round of PCR amplification (nest-PCR) after the initial RT-PCR with CSF-1R3 and CSF-1R6. The

identity of the nested-PCR product was confirmed by diagnostic digestion with a restriction enzyme *Alu* I, which was predicted to have a single cutting site within the 303-nt porcine sequence (Fig. 2.12F). Expression of c-fms mRNA was detected in higher levels in small blastocysts (1-2 mm in diameter) than in medium-sized blastocysts (5-6 mm), and progressively increased in both large blastocysts (8-10 mm) and elongated blastocysts obtained between Days 11 to 15.75 of pregnancy (Fig. 2.12E). Expression of c-fms mRNA therefore exhibits a similar pattern to its ligand, albeit at much lower levels, in blastocysts during peri-implantation development (Fig. 2.12C).

DISCUSSION

The stimulatory activity of conditioned media of Jag-1 cells, shown in the *in vitro* proliferation assay, suggests that Jag-1 cells could produce a soluble factor capable of stimulating the proliferation of porcine macrophages. This result is consistent with a previous report by Ramsoondar et al. (1993). This factor is present in conditioned serum-free medium harvested from Jag-1 trophoblast cells and as such represents a potential autocrine/ paracrine growth factor. The anti-human CSF-1 neutralizing monoclonal antibody YY106 can specifically inhibit the stimulatory activity in Jag-1 cell-conditioned medium, and also the proliferation of Jag-1 cells themselves, which provides strong evidence that CSF-1 is the bioactive growth factor produced by JAG-1 trophoblast cells. Taken together, these results suggest that porcine CSF-1 is an important aurocrine/paracrine growth factor for stimulating proliferation of pre-implantation trophoblast cells. These observations of a potential role for CSF-1 in regulating

proliferation of embryonic tissue(s) was the basis for fully characterizing the gene encoding porcine CSF-1 and its expression in embryonic and endometrial tissues.

Using a RT-PCR approach, a porcine CSF-1 cDNA has been cloned from Jag-1 cells derived from porcine embryos at Day 14 of pregnancy (Ramsoondar et al., 1993). The coding sequence of porcine CSF-1 shows an overall 82% and 73% sequence identity with those of human and mouse CSF-1, respectively, at the nucleotide level. In an earlier study (Tuo et al., 1995), a partial coding sequence (823 nt) of porcine CSF-1 was obtained from Day 15 porcine embryos, and was reported to have greater than 98% identity with the human counterpart (sequence data was not published or submitted to the GenBank database). However, the corresponding 823-nt sequence characterized in the present study shows 87% sequence identity to the human counterpart. The reason for this discrepancy is unknown.

Using the porcine CSF-1 cDNA clone as a probe, one major mRNA species of about 4 kb was detected in Jag-1 cells and all endometrial tissues examined by Northern blot analysis. In addition, two extra mRNA species of about 5.1 kb and 3.6 kb, respectively, were also detected in endometrial tissues around Day 16 of pregnancy but not in Jag-1 cells. These results are consistent with the findings in a previous study (Tuo et al., 1995), where the two mRNA species of 5.1 kb and 3.6 kb were detected in porcine endometrial and oviductal tissues at Day 15 but not in embryonic tissues at Day 15 by Northern hybridization analysis, using a human CSF-1 cDNA probe. Similar transcript size heterogeneity occurs in many types of human tissues and cells, including pregnant endometrium and placenta, monocytes, endothelial cells, fibroblasts and keratinocytes, where the 4-kb CSF-1 mRNA species was also found to be predominantly expressed

(Daiter et al., 1992; Stanley, 1990; Seelentag et al., 1987; Akashi et al., 1989; Chodakewitz et al., 1990). In the mouse, the predominant CSF-1 mRNA species was found to be 2.3 kb in length in pregnant uterus, although it was not detectable in nongravid uterus, in which the 4-kb species was predominantly expressed (Arceci et al., 1989).

The present study demonstrates that porcine endometrial CSF-1 mRNA expression reaches a peak on Day 3 of pregnancy, when embryos are still in the oviduct. It is known that following insemination the majority of sperm remain in the uterus on their way towards the fertilization site in the oviduct. CSF-1 has been shown to act as a chemotactic factor for monocytes and to promote the proliferation and differentiation of macrophages (Sherr and Stanley, 1990; Stanley, 1990). Observations in rodents suggest that variations in the uterine macrophage population might be an indirect effect of the release of CSF-1 by the glandular epithelium (De and Wood, 1990; Pollard et al., 1991). In addition, it has been demonstrated in the mouse that the acute accumulation of uterine macrophages on Days 1 and 2 of pregnancy occurs coincident with peak expression of CSF-1 in the uterus, which functions primarily in the elimination of sperm from the uterus (Wood et al., 1997). Therefore, peak CSF-1 expression around Day 3 in the pig might have its primary function in recruiting monocytes and promoting proliferation and differentiation of uterine macrophages in order to eliminate the large number of sperm remaining in the uterine lumen. Thereafter, an appropriate uterine environment could be established for subsequent embryo development in the uterus.

In this study, porcine endometrial CSF-1 mRNA expression level was elevated by about 3-fold on Day 16 in comparison to basal levels observed between Days 4 and

15.5. CSF-1 mRNA levels were also up regulated around Day 16 of pregnancy in embryonic tissues, although at much lower absolute levels. The lower levels of embryo-derived CSF-1 in samples obtained from pigs between Days 9 and 15.75 of pregnancy indicate that the endometrium might be a more important source of CSF-1 during pre-implantation development of porcine embryos. In contrast, the allantochorionic placenta was found to express a higher level of CSF-1 than the endometrium during the post-implantation period in the pig (Tuo et al., 1995). More importantly, mRNA expression for both CSF-1 and c-fms were detected and exhibited similar temporal patterns in porcine embryos during peri-implantation development. In addition, CSF-1 was shown to be the major autocrine factor regulating the proliferation of trophoblast cells *in vitro* in the present study. Taken together, these results suggest that CSF-1 might act in both autocrine and paracrine fashion on the proliferation of porcine trophoblast cells during the pre-implantation period, which is in contrast to only a paracine role of CSF-1 on mouse pre-implantation embryos (Arceci et al., 1992).

Over-expression of c-fms was shown to facilitate cellular invasion and anchorage-independent growth in mammary epithelial cells, and to reduce the concentration of retinoic acid or 1,25-dihydroxy vitamin D3 needed to cause monocytic cell differentiation (Sapi et al., 1996; Yen et al., 1997). In addition, c-fms was shown to be expressed at very high levels on placental trophoblasts, especially invasive trophoblast (reviewed by Hume et al., 1997; Aboagye-Mathiesen et al., 1997; Pampfer et al., 1992). However, expression of c-fms mRNA was observed at very low levels in pre-implantation porcine blastocysts in the present study. Therefore, we could speculate that the expression level of c-fms in porcine trophoblast cells might be not enough to induce

the differentiation of trophoblast cells that is essential for the acquisition of invasiveness by trophoblast cells during embryo implantation as seen in humans and mice. As a result, trophoblast cells could not acquire the ability *in vivo* to invade the endometrium during embryo implantation in pigs, although they appeared to be invasive after they were transferred to ectopic sites (Samuel, 1971 & 1972; Samuel and Perry, 1972).

Our Southern blot analysis indicates that the porcine CSF-1 gene presents as a single copy in the genome. In conjunction with previous studies, our results suggest that porcine CSF-1 might be very similar to human and mouse CSF-1 in gene structure and its expression regulation during pregnancy. The present study will certainly facilitate further studies on the structure and expression regulation of the porcine CSF-1 gene and on its potential roles in porcine implantation and placental development.

It should be noted that the last 18 nucleotides of the coding sequence of porcine CSF-1 are not completely derived from porcine CSF-1 mRNA, since the down-stream primer CSF-1D is designed from the conserved region between human and mouse sequences. However, we may assume that it is very similar between pig, human and mouse sequences in this region. To confirm the functionality of the cloned porcine CSF-1 cDNA sequence, we have introduced the full-length coding sequence of porcine CSF-1 into the genome of COS-7 cells through an eukaryotic expression vector and are currently evaluating the activity of the CSF-1 in a sensitive bioassay.

Table 2.1 Sequences of CSF-1 and c-fms primers designed in this experiment [a]

Primer	Sequence [b] (Shown as 5' → 3')	Annealing temperature used for PCR amplification
CSF-1S	TGAATTCCCAGCTGCCCCGTATG	58 °C
CSF-15	TGCCCCGTATGACCGCGCCG	
CSF-13	GTCTCCATCTGACTGTCAATCAGCTG	
CSF-1U	TGAATTCACTGTAGCCACATGATTGGG	65 °C
CSF-1D	TAGATCTCTACACTGGCAGTTCCAC	
PR3	CTCAGCAGAACTGATAGTTGTTGGGCTGC	65 °C
PR6	AACTGGTGAAGGATGGATACCAAATGGC	
PR7	TACAGCATCATGCAGGCCTGCTGGG	60 °C
PR8	ATCCCCCTGCTCGCAGCAGGCCA	
G3PDH-U	ACCACAGTCCATGCCATCAC	60 °C
G3PDH-D	TCCACCACCCTGTTGCTGTA	
Actin-U	ATCTTGATCTTCATGGTGCTGGGC	
Actin-D	ACCACTGGCATTGTCATGGACTCT	

[a] Locations and directions of primers are shown in Figure 5 and Figure 12.

[b] A restriction site (underlined) is attached to some of the primers.

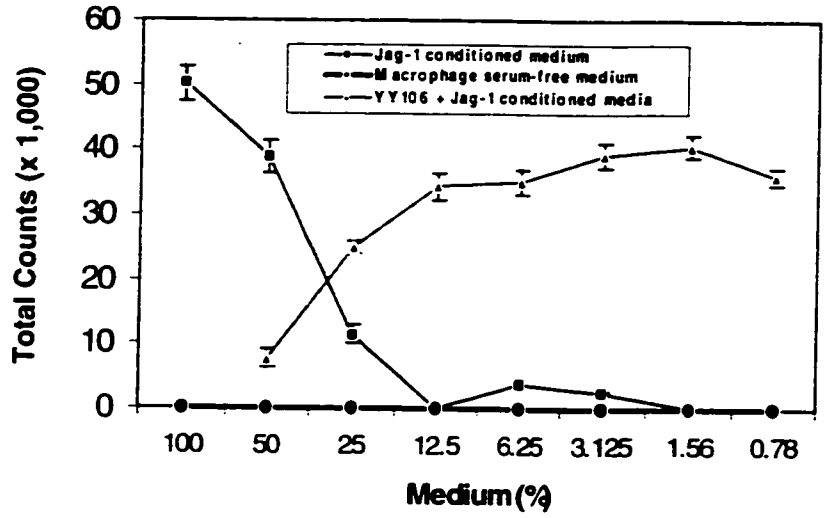
Table 2.2 The homologies of the coding sequences and deduced amino acid sequences of porcine, human and mouse CSF-1[a], and the positions of potential N-linked glycosylation (N-X-S/T) and glycosaminoglycan addition (acidic residues-S-G-X-G/A)

	Homology[b] of the coding sequence (deduced **amino acid sequence**)			Positions[c] of N-X-S/T	Positions of glycosaminogly-can addition site
	H CSF-1	M CSF-1	P CSF-1		
P CSF-1	82% (**73%**)			38;**154**;**172**	309
H CSF-1		77% (**67%**)		**154**;**172**;381;415	309
M CSF-1			73% (**65%**)	107;**154**,**172**,378	308

[a] Rat CSF-1 is not included because its coding sequence is identical to that of mouse CSF-1 (Borycki et al., 1993), and the short forms (256aa and 438aa) of human CSF-1 (Kawasaki et al., 1985) are also not included here. [b] The data were obtained with the aid of a computer program (GeneJockey II). [c] Amino acids were counted by referring the position of starting methionine as the first one.

Figure 2.1 A). Stimulatory effect (■) of Jag-1 cell-conditioned medium (CM) and inhibitory effect (▲) of YY106 hybridoma CM on the proliferation of porcine monocyte/macrophages. Peak stimulation occurred at 100% Jag-1 CM. The stimulatory effect in 50% Jag-1 CM was blocked in a dose-dependent fashion by YY106 hybridoma CM. Macrophage serum-free medium was used as a control (●) in this experiment. B). Inhibitory effect of YY106 hybridoma CM on the proliferation of Jag-1 cells was in a dose-dependent fashion. Results were presented as the mean ± SEM of six reaction replicates.

A


Jag-1 conditioned medium
Macrophage serum-free medium
YY106 + Jag-1 conditioned media
Total Counts (x 1,000)
60
50
40
30
20
10
0
100
50
25
12.5
6.25
3.125
1.56
0.78
Medium (%)

B

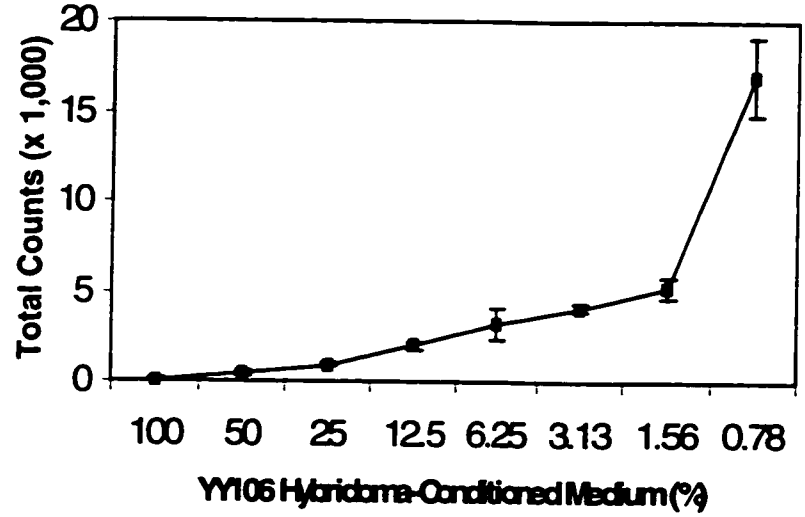
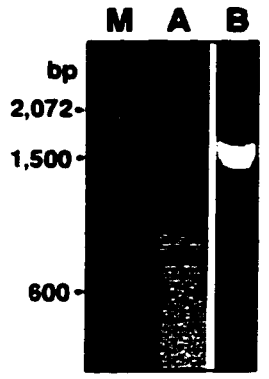
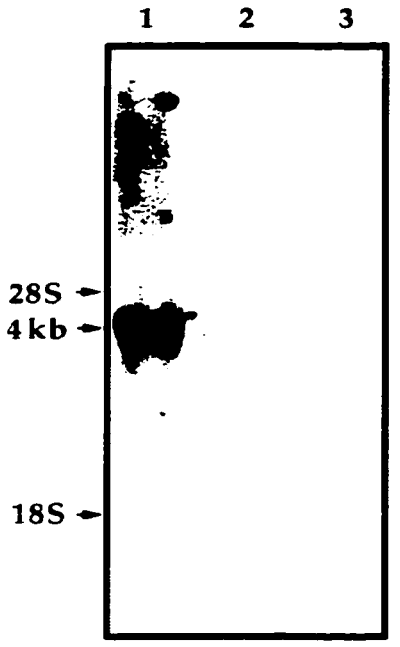

Total Counts (x 1,000)
20
15
10
5
0
100
50
25
12.5
6.25
3.13
1.56
0.78
YY106 Hybridoma-Conditioned Medium (%)

Figure 2.2 (Top) The amplification of the 1,569-bp cDNA fragment from mRNA of Jag-1 cells. Lane A, one fifth of the products from RT-PCR, which was carried out using total RNA (1μg) of Jag-1 cells and the primer pair CSF-1U & CSF-1D, was fractionated in 0.8% (w/v) agarose gel. Lane B, the amplicon of 1,569 bp was isolated and re-amplified under the same conditions and with the same primer pair as in RT-PCR. One fifth of the PCR product was run in 0.8% (w/v) agarose gel. Both of the gels were stained with ethidium bromide. Lane M, 100-bp DNA ladder of 0.5 μg.

Figure 2.3 (Bottom left) Northern blot hybridization analysis of RNA extracted from Jag-1, H69B and H69C cells. The 1,569-bp cDNA fragment of porcine CSF-1 gene was used as the probe. Twenty micrograms of total RNA from Jag-1 (Lane 1), H69B (Lane 2) and H69C (Lane 3) cells was denatured and fractionated on a 1% (w/v) agarose gel containing 7% (v/v) formaldehyde in MOPS buffer.

Figure 2.4 (Bottom right) RT-PCR and restriction analysis of the 5' end region of porcine CSF-1 coding sequence. RT was done under the same conditions as described in Fig. 2.2, and PCR was carried out with the primer pair CSF-1S & CSF-13. Electrophoresis was done in 1.3% (w/v) agarose gel, and the gel was stained in ethidium bromide. Lane M, 100-bp DNA ladder. Lane A, RT-PCR products of 8 μl. Lane B, restriction digests of the RT-PCR products of 10 μl with *Pst* I (37 °C, 1 hr).


M A B
bp
2,072
1,500
600


1 2 3
28S
4kb
18S

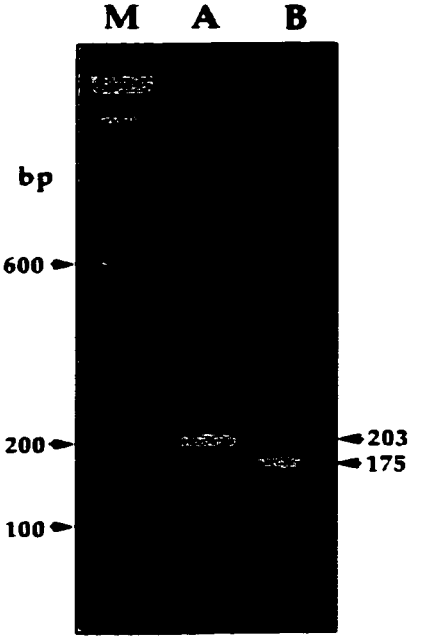
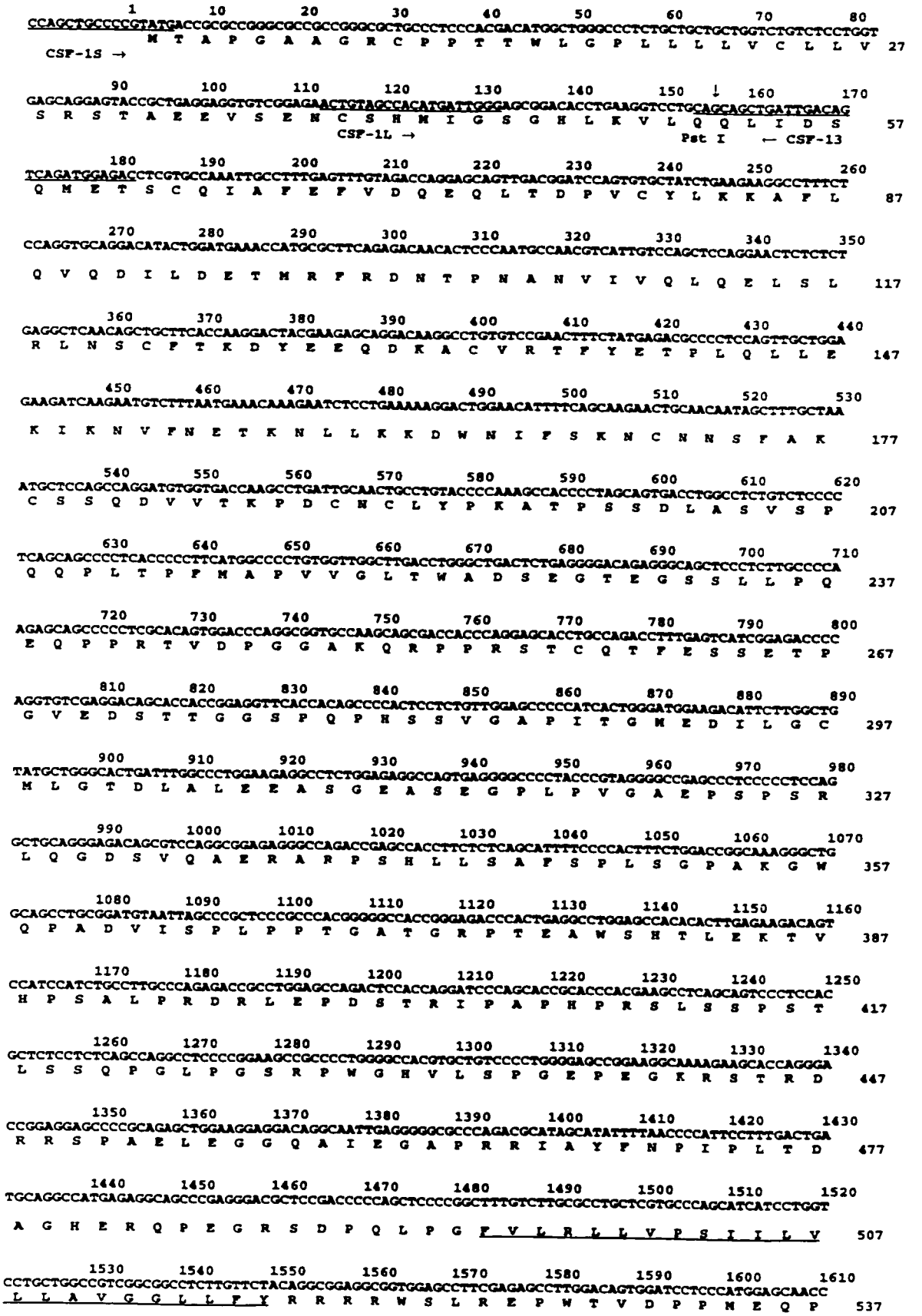

M A B
bp
600
200
100
203
175

Figure 2.5 Nucleotide sequence and deduced amino acid sequence of porcine CSF-1. The numberings of the nucleotides and amino acids begin at the start methionine code and are indicated above and to the right of their corresponding sequences, respectively. The positions of the primers used for PCR in this study are underlined under the nuclectide sequence, with arrows indicating their priming directions. The transmembrane domain is underlined at the bottom of the amino acid sequence. The *Pst* I restriction site at the 5' end is indicated with a vertical arrow.


CSF-1S →
CSF-1L →
Pst I
← CSF-13

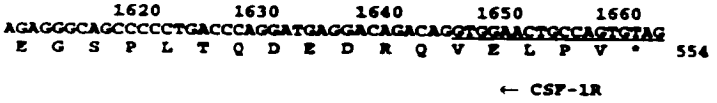

1620
1630
1640
1650
1660
AGAGGGCAGCCCCCTGACCCAGGATGAGGACAGACAGGTGGAACTGCCAGTGTAG
E G S P L T Q D E D R Q V E L P V *
554
← CSF-1R

Figure 2.6 Comparison of the amino acid sequences of porcine, human and mouse CSF-1. The sequence alignment was done with the GeneJockey II computer program. The bullet mark (•) indicates a perfect agreement between sequences. Potential N-linked glycosylation sites are underlined with solid lines, and the glycosaminoglycan addition site is underlined with a dotted line. The transmembrane domain is indicated with a double dotted line.

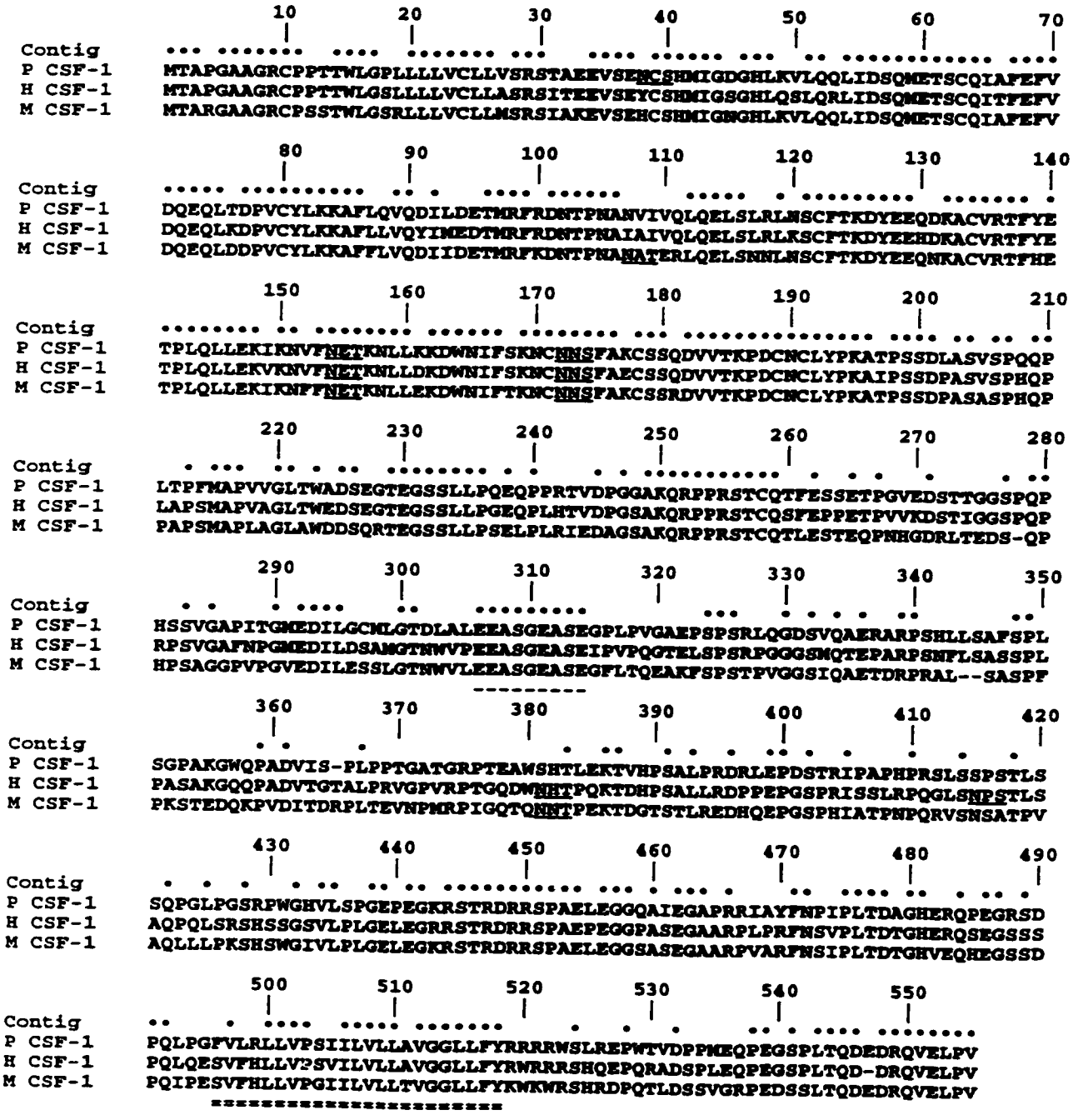

10 20 30 40 50 60 70
Contig
P CSF-1 MTAPGAAGRCPPTTWLGPLLLLVCLLVSRSTAEEVSEECSHMIGDGHLKVLQQLIDSQMETSCQIAFEFV
H CSF-1 MTAPGAAGRCPPTTWLGSLLLLVCLLASRSITEEVSEYCSHMIGSGHLQSLQRLIDSQMETSCQITFEFV
M CSF-1 MTARGAAGRCPSSTWLGSRLLLVCLLMSRSIAKEVSEHCSHMIGNGHLKVLQQLIDSQMETSCQIAFEFV
80 90 100 110 120 130 140
Contig
P CSF-1 DQEQLTDPVCYLKKAFLQVQDILDETMRFRDNTPNAIVIVQLQELSLRLNSCFTKDYEEQDKACVRTFYE
H CSF-1 DQEQLKDPVCYLKKAFLLVQYIMEDTMRFRDNTPNAIAIVQLQELSLRLKSCFTKDYEEHDKACVRTFYE
M CSF-1 DQEQLDDPVCYLKKAFFLVQDIIDETMRFKDNTPNANATERLQELSNNLNSCFTKDYEEQNKACVRTFHE
150 160 170 180 190 200 210
Contig
P CSF-1 TPLQLLEKIKNVFNETKNLLKKDWNIFSKNCNNSFAKCSSQDVVTKPDCNCLYPKATPSSDLASVSPQQP
H CSF-1 TPLQLLEKVKNVFNETKNLLDKDWNIFSKNCNNSFAECSSQDVVTKPDCNCLYPKAIPSSDPASVSPHQP
M CSF-1 TPLQLLEKIKNFFNETKNLLEKDWNIFTKNCNNSFAKCSSRDVVTKPDCNCLYPKATPSSDPASASPHQP
220 230 240 250 260 270 280
Contig
P CSF-1 LTPFMAPVVGLTWADSEGTEGSSLLPQEQPPRTVDPGGAKQRPPRSTCQTFESSETPGVEDSTTGGSPQP
H CSF-1 LAPSMAPVAGLTWEDSEGTEGSSLLPGEQPLHTVDPGSAKQRPPRSTCQSFEPPETPVVKDSTIGGSPQP
M CSF-1 PAPSMAPLAGLAWDDSQRTEGSSLLPSELPLRIEDAGSAKQRPPRSTCQTLESTEQPNHGDRLTEDS-QP
290 300 310 320 330 340 350
Contig
P CSF-1 HSSVGAPITGMEDILGCMLGTDLALEEASGEASEGPLPVGAEPSPSRLQGDSVQAERARPSHLLSAFSPL
H CSF-1 RPSVGAFNPGMEDILDSAMGTNWVPEEASGEASEIPVPQGTELSPSRPGGGSMQTEPARPSNFLSASSPL
M CSF-1 HPSAGGPVPGVEDILESSLGTNWVLEEASGEASEGFLTQEAKFSPSTPVGGSIQAETDRPRAL--SASPF
----
360 370 380 390 400 410 420
Contig
P CSF-1 SGPAKGWQPADVIS-PLPPTGATGRPTEAWSHTLEKTVHPSALPRDRLEPDSTRIPAPHPRSLSSPSTLS
H CSF-1 PASAKGQQPADVTGTALPRVGPVRPTGQDWNHTPQKTDHPSALLRDPPEPGSPRISSLRPQGLSNPSTLS
M CSF-1 PKSTEDQKPVDITDRPLTEVNPMRPIGQTQNNTPEKTDGTSTLREDHQEPGSPHIATPNPQRVSNSATPV
430 440 450 460 470 480 490
Contig
P CSF-1 SQPGLPGSRPWGHVLSPGEPEGKRSTRDRRSPAELEGGQAIEGAPRRIAYFNPIPLTDAGHERQPEGRSD
H CSF-1 AQPQLSRSHSSGSVLPLGELEGRRSTRDRRSPAEPEGGPASEGAARPLPRFNSVPLTDTGHERQSEGSSS
M CSF-1 AQLLLPKSHSWGIVLPLGELEGKRSTRDRRSPAELEGGSASEGAARPVARFNSIPLTDTGHVEQHEGSSD
500 510 520 530 540 550
Contig
P CSF-1 PQLPGFVLRLLVPSIILVLLAVGGLLFYRRRRWSLREPWTVDPPMEQPEGSPLTQDEDRQVELPV
H CSF-1 PQLQESVFHLLVPSVILVLLAVGGLLFYRWRRRSHQEPQRADSPLEQPEGSPLTQD-DRQVELPV
M CSF-1 PQIPESVFHLLVPGIILVLLTVGGLLFYKWKWRSHRDPQTLDSSVGRPEDSSLTQDEDRQVELPV
==========

Figure 2.7 Southern blot hybridization analysis of Jag-1 cell DNA digested with restriction enzymes, *Bam* HI (Lane A), *Eco* RI (Lane B), *Hind* III (Lane C) and *Pst* I (Lane D), respectively. The blot was hybridized with the same probe DNA as used for Northern blot hybridization (Fig. 2.3), except that the labeled probe DNA was separated from unincorporated α-^{32}p-dATP before adding to the hybridization buffer.

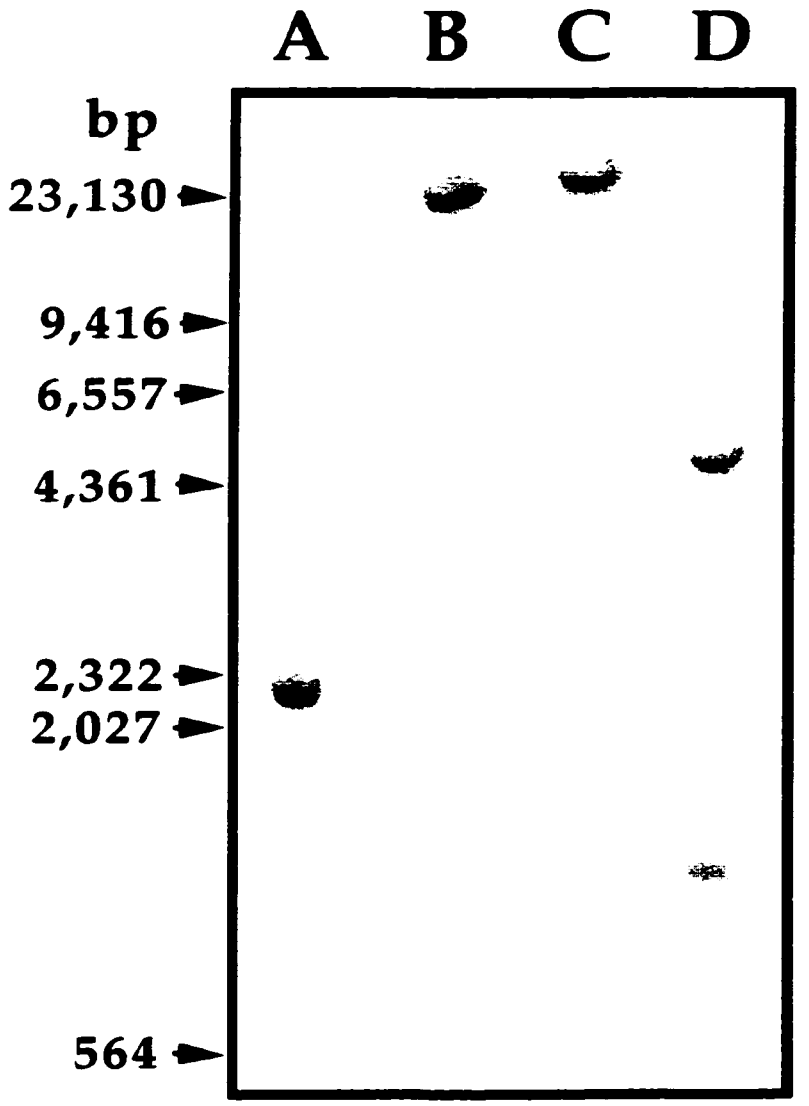
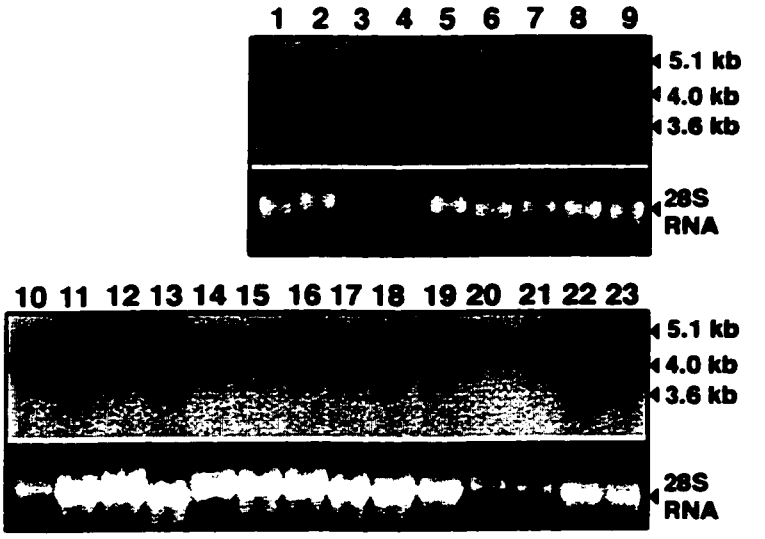

A
B
C
D
bp
23,130
9,416
6,557
4,361
2,322
2,027
564

Figure 2.8 (Upper two panels) Northern hybridization analysis of RNA extracted from endometrial tissues during pre-implantation development. Lanes 1 to 22 represent endometrial RNA samples from 21 individual pigs on Days 9.5, 11, 11.75, 12, 12, 13.5, 15.5, 15.75, 15.75, 3, 3, 4, 5.5, 5.5, 7, 7.5, 9, 9.5, 10.75, 12.75, 13.75, 16.5 of pregnancy, respectively. Samples in Lanes 1 and 19 are from the same Day 9.5 pig. Lane 23 is RNA from Jag-1 cells which was used as a positive control. In the upper panel are Northern hybridization signals, and in the lower panel are the corresponding 28S RNA amounts on the nitrocellulose membrane after transfer from the agarose gel, which are used as the internal control of RNA loading for quantitative analysis. The 1,569-bp cDNA fragment of porcine CSF-1 gene was used as the probe.

Figure 2.9 (Lower panel) Quantitative analysis of the 4-kb CSF-1 mRNA expression in endometrial tissues between Day 3 and Day 16.5. Data for CSF-1 mRNA levels and 28S RNA levels were obtained using the Imaging Densitometer GS-670 (BioRad) and the Gel Doc 1000 (BioRad) with the aid of the Molecular Analyst Software (Version 2.1) (BioRad, California). Expression levels of the 4-kb CSF-1 mRNA in each of endometrial samples were normalized as percentages of their corresponding 28S RNA levels on the nitrocellulose membranes.


1 2 3 4 5 6 7 8 9
5.1 kb
4.0 kb
3.6 kb
28S RNA
10 11 12 13 14 15 16 17 18 19 20 21 22 23
5.1 kb
4.0 kb
3.6 kb
28S RNA

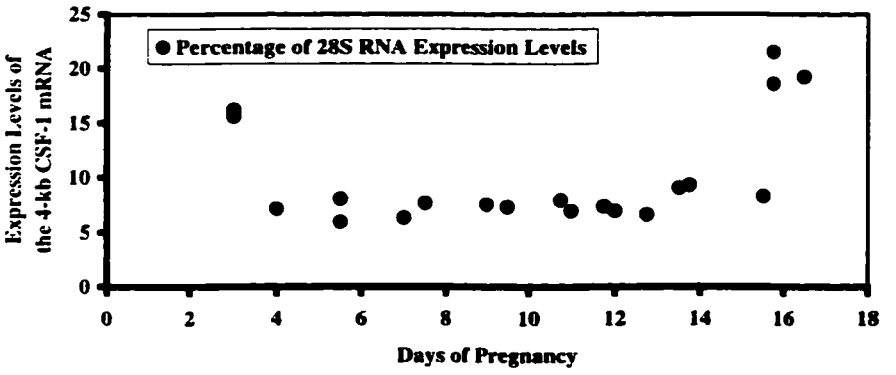

Percentage of 28S RNA Expression Levels
Expression Levels of the 4-kb CSF-1 mRNA
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
Days of Pregnancy

Figure 2.10 Comparison of CSF-1 mRNA expression levels between endometrial and embryonic tissues during pre-implantation development. Lanes 1 to 7 represent 7 individual pigs at Days 11, 11.75, 12, 13.5, 15.5; 15.75 and 15.75 of pregnancy, respectively. Lane T, Jag-1 cells. Both endometrial and embryonic RNA were isolated from each of these pigs. Using RT-PCR, primers CSF-15 and CSF-13 were used to amplify the 5' end 197-bp sequence of porcine CSF-1 including the deduced signal sequence, which is common to all cDNA sequences of human CSF-1 identified so far. CSF-1 PCR products were confirmed by restriction digestion with Pst I as in Fig. 4. RNA from Jag-1 cells was used as a positive control, and β-actin and G3PDH were co-amplified in all RNA samples as internal controls of gene expression.

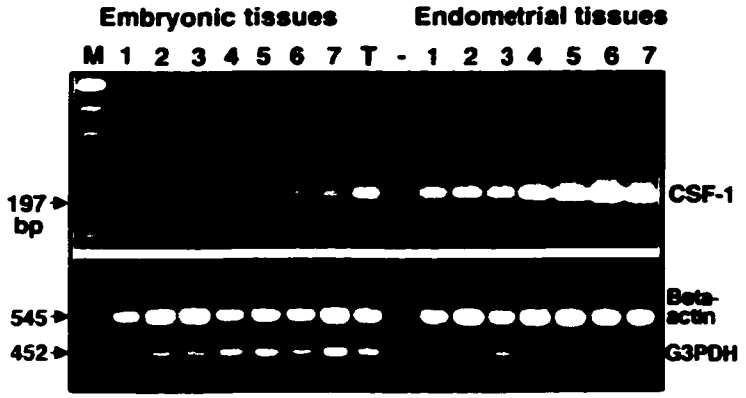

Embryonic tissues
Endometrial tissues
M 1 2 3 4 5 6 7 T - 1 2 3 4 5 6 7
197 bp
CSF-1
545
452
Beta-actin
G3PDH

Figure 2.11 A). Nucleotide sequence of the 303-bp fragment of porcine c-fms cDNA. The primer sequences and directions are indicated with underlines and arrows, respectively. The termination code is boxed. B). Comparison of the deduced 112 amino acid sequences of porcine, human and mouse c-fms. The bullet mark (•) indicates a perfect agreement between sequences. The variable region between sequences is indicated within two vertical slashes.

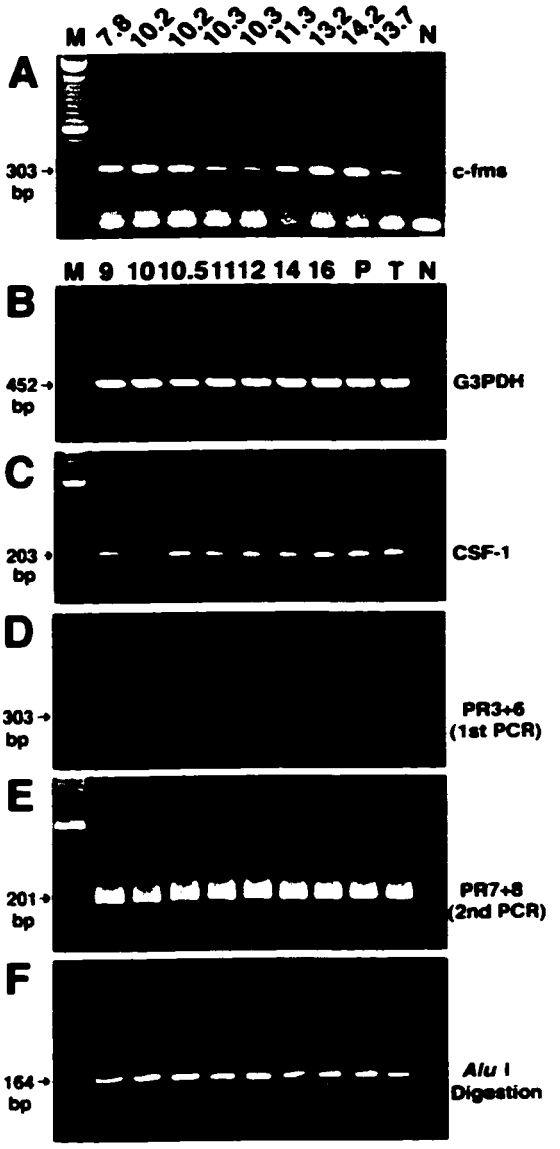
A

```
                                PR6→
AACTGGTGAA GGATGGATAC CAAATGGCCC AGCTGGCGTT CGCCCCCAAG AACATATACA
                          PR7→
GCATCATGCA GGCCTGCTGG GCCCTGGAGC CGACGCGCAG ACCCACCTTC CAGCAGATCT

GCTCCCTCCT TCAGGAGCAG AGCCACGGCG ACCAGAGAGC GCGGGACTAC AGCAACCTGC
                                      AluI I↓                ←PR8
CGAGCAGCAG CAGCAGCAGC AGCGAGCCCG AGGAGGGGAG CTCCGGCGAG CACCTGGCCT
                                     ←PR3
GCTGCGAGCA GGGGGATGCG GCCCAGCCCC TGCTGCAGCC CAACAACTAT CAGTTCTGCT GAG
```

B

```
                  10         20         30         40         50
                  |          |          |          |          |
Contig    ••••••••••  •••••••••• •• •••• ••••••••  ••••     •  •• ••
Human     LVKDGYQMAQPAFAPKNIYSIMQACWALEPTHRPTFQQICSFLQEQAQEDRRERDYTNL
Mouse     LVKDGYQMAQPVFAPKNIYSIMQSCWDLEPTRRPTFQQICFLLQEQARLERRDQDYANL
Pig       LVKDGYQMAQLAFAPKNIYSIMQACWALEPTRRPTFQQICSLLQEQSHGDQRARDYSNL

          60         70         80         90         100        110
          |          |          |          |          |          |
Contig    •••|              |•••• •• •• ••• ••• •• ••••••••••••
Human     PSSSRSGGS-------GSSSSELEEESSSEHLTCCEQGDIAQPLLQPNNYQFC•
Mouse     PSSGGSSGSDSGGGSSGGSSSEPEEESSSEHLACCEPGDIAQPLLQPNNYQFC•
Pig       PSSS-------------SSSSEPEEGSSGEHLACCEQGDAAQPLLQPNNYQFC•
```

Figure 2.12 A). RT-PCR amplification (45 cycles) of c-fms mRNA in porcine endometrium. Lanes 1-9 represent endometrial RNA samples obtained from individual pigs on Day 9.5, 11, 11.75, 12, 12, 13.5, 15.5, 15.75 and 15.75 of pregnancy, respectively. B-F). PCR amplification of CSF-1 and c-fm mRNA in porcine pre-implantation blastocysts [RT-PCR of G3PDH (B), CSF-1 (C), and c-fms (D); Nest-PCR of c-fms (E); Diagnostic digestion of nest-PCR products in gel E with *Alu* I (F)]. M, 100-bp DNA ladder. Samples in gels B-F were spherical blastocysts of 1-2 mm (Lane 9), 5-6 mm (Lane 10) and 8-10 mm (Lane 10.5) in diameter and elongated blastocysts obtained on Day 11 (Lane 11), 12 (Lane 12), 14 (Lane 14) and 16 (Lane 16) of pregnancy, respectively. Electrophoresis was carried out in 1.2 % agarose gel, and gels were stained with ethidium bromide.


M 7.8 10.2 10.2 10.3 10.3 11.3 13.2 14.2 13.7 N
A
303 → bp
c-fms
M 9 1010.51112 14 16 P T N
B
452 → bp
G3PDH
C
203 → bp
CSF-1
D
303 → bp
PR3+6 (1st PCR)
E
201 → bp
PR7+8 (2nd PCR)
F
164 → bp
Alu I Digestion

REFERENCES

Aboagye-Mathiesen G, Zdravkovic M, Toth FD, Ebbesen P (1997): Effects of human trophoblast-induced interferons on the expression of proto-oncogenes c-fms/CSF-1R, EGF-R and c-erbB2 in invasive and non-invasive trophoblast. Placenta 18(2-3):155-161.

Akashi M, Saito M, Koeffler HP (1989): Lymphotoxin: stimulation and regulation of colony-stimulating factors in fibroblasts. Blood 74(7): 2383-2390.

Arceci RJ, Pampfer S, Pollard JW (1992): Expression of CSF-1/c-fms and SF/c-kit mRNA during preimplantation mouse development. Dev. Biol. 151(1):1-8.

Arceci RJ, Shanahan F, Stanley ER, Pollard JW (1989): Temporal expression and location of colony-stimulating factor 1 (CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1-regulated placental development. Proc. Natl. Acad. Sci. USA 86(22): 8818-8822.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994): "Current Protocols in Molecular Biology." New York: John Wiley & Sons Inc.

Bartocci A, Pollard JW, Stanley ER (1986): Regulation of colony-stimulating factor 1 during pregnancy. J. Exp. Med. 164(3): 956-961.

Bazer FW (1994): New frontiers and approaches in the study of embryonic mortality in domestic species. In Zavy MT, Geisert RD (ed): "Embryonic Mortality in Domestic Species." Boca Raton: CRC Press, pp 195-210.

Borycki A, Lenormand J, Guillier M, Leibovitch SA (1993): Isolation and characterization of a cDNA clone encoding rat CSF-1 gene. Post-transcriptional repression occurs in myogenic differentiation. Biochem. Biophys. Acta 1174: 143-

152.

Cerretti DP, Wignall J, Anderson D, Tushinski RJ, Gallis BM, Stya M, Gillis S, Urdal DL, Cosman D (1988): Human macrophage colony-stimulating factor: alternative RNA and protein processing from a single gene. Mol. Immunol. 25: 761-770.

Chodakewitz JA, Lacy J, Edwards SE, Birchall N, Coleman DL (1990): Macrophage colony-stimulating factor production by murine and human keratinocytes. Enhancement by bacterial lipopolysaccharide. J. Immunol. 144(6): 2190-2196.

Cohen PE, Zhu L, Pollard JW (1997): Absence of colony-stimulating factor-1 in osteopetrotic ($csfm^{op}/csfm^{op}$) mice disrupts estrous cycles and ovulation. Biol. Reprod. 56: 110-118.

Cohen PE, Chisholm O, Arceci RJ, Stanley ER, Pollard JW (1996): Absence of colony-stimulating factor-1 in osteopetrotic ($csfm^{op}/csfm^{op}$) mice results in male fertility defects. Biol. Reprod. 55(2): 310-317.

Coussens L, Van Beveren C, Smith D, Chen E, Mitchell RL, Isacke CM, Verma IM, Ullrich A (1986): Structural alteration of viral homologue of receptor proto-oncogene fms at carboxyl terminus. Nature 320 (6059), 277-280.

Daiter E, Pampfer S, Yeung YG, Barad D, Stanley ER, Pollard JW (1992): Expression of colony-stimulating factor-1 in the human uterus and placenta. J. Clinical Endocrinol. & Metabol. 74(4): 850-858.

De M, Wood GW (1990): Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. J. of Endocrinol. 126(3): 417-424.

Deng P, Wang YL, Pattengale PK, Rettemier CW (1996): The role of individual cysteine residues in the processing, structure, and function of human macrophage colony-stimulating factor. Biochem. and Biophys. Res. Commun. 228: 557-566.

Dixon WT, Varedi M, Meuser R, Searles GE, Shaw ARE (1995): Differentiation *in vitro* of an invasive small cell lung carcinoma cell line leads to acquisition of an adherent, non-tumorigenic phenotype. J. Cellular Biochem. 19B: 14.

Feyles V, Sikora LK, McGarry RC, Jerry LM (1991): Effects of retinoic acid and bromodeoxyuridine on human melanoma-associated atigen expression in small cell lung carcinoma cells. Oncology 48(1): 58-64.

Filderman AE, Bruckner A, Kacinski BM, Deng N, Remold HG (1992): Macrophage colony-stimulating factor (CSF-1) enhances invasiveness in CSF-1 receptor-positive carcinoma cell lines. Cancer Res. 52(13): 3661-3666.

Fixe P, Praloran V (1997): Macrophage colony-stimulating-factor (M-CSF or CSF-1) and its receptor: structure-function relationships. Eur. Cytokine Netw. 8(2): 125-136.

Hume DA, Yue X, Ross IL, Favot P, Lichanska A, Ostrowski MC (1997): Regulation of CSF-1 receptor expression. Mol. Reprod. & Dev. 46(1): 46-52.

Groffen J, Heisterkamp N, Spurr N, Dana S, Wasmuth JJ, Stephenson JR (1983): Chromosomal localization of the human c-fms oncogene. Nucleic Acids Res. 11(18):6331-6339.

Kacinski BM (1997): CSF-1 and its receptor in breast carcinomas and neoplasmas of the female reproductive tract. Mol. Reprod. and Dev. 46(1): 71-74.

Kawasaki ES, Ladner MB, Wang AM, Arsdell JV, Warren MK, Coyne MY, Schweickart VL, Lee MT, Wilson KJ, Boosman A, Stanley ER, Ralph P, Mark DF (1985):

Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). Science 230: 291-296.

Kawasaki ES, Ladner MB (1990): Molecular biology of macrophage colony-stimulating factor. In Dexter, T.M., Garland, J.M., Testa, N.G. (Eds.), Colony-Stimulating Factors: Molecular and Cellular Biology, Marcel Dekker Inc., New York, USA, pp. 475.

Ladner MB, Martin GA, Noble JA, Nikoloff DM, Kawasaki ES, White TJ (1987): Human CSF-1: gene structure and alternative splicing of mRNA precursors. EMBO J. 6: 2693-2698.

Ladner MB, Martin GA, Noble JA, Wittman VP, Warren MK, McGrogan M, Stanley E R (1988): cDNA cloning and expression of murine macrophage colony-stimulating factor from L929 cells. Proc. Natl. Acad. Sci. USA 85(18): 6706-6710.

Pampfer S, Daiter E, Barad D, Pollard JW (1992): Expression of the colony-stimulating factor-1 receptor (c-fms proto-oncogene product) in the human uterus and placenta. Biol. Reprod. 46(1):48-57.

Pampfer S, Arceci RJ, Pollard JW (1991): Role of colony stimulating factor-1 (CSF-1) and other lympho-hematopoietic growth factors in mouse pre-implantation development. Bioessays 13(10): 535-540.

Pampfer S, Tabibzadeh S, Chuan FC, Pollard JW (1991): Expression of colony-stimulating factor-1 (CSF-1) messenger RNA in human endometrial glands during the menstrual cycle: molecular cloning of a novel transcript that predicts a cell surface form of CSF-1. Mol. Endocrinol. 5(12): 1931-1938.

Pettenati MJ, LeBeau MM, Lemons RS, Shima EA, Kawasaki ES, Larson RA, Sherr CJ, Diaz MO, Rowley JD (1987): Assignment of CSF-1 to 5q 33.1: evidence for

clustering of genes regulating hematopoiesis and for their involvement in the deletion of the long arm of chromosome 5 in myeloid disorders. Proc. Natl. Acad. Sci. USA 84: 2970-2974.

Pollard JW (1997): Role of colony-stimulating factor-1 in reproduction and development. Mol. Reprod. & Dev. 46(1): 54-60.

Pollard JW, Hennighausen L (1994): Colony stimulating factor 1 is required for mammary gland development during pregnancy. Proc. Natl. Acad. Sci. USA 91(20): 9312-9316.

Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER (1991): A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev. Biol. 148(1): 273-283.

Pollard JW (1990): Regulation of polypeptide growth factor synthesis and growth factor-related gene expression in the rat and mouse uterus before and after implantation. J. Reprod. & Fertility 88(2): 721-731.

Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB, Stanley ER (1987): Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature 330 (6147): 484-486.

Rajavashisth TB, Eng R, Shadduck RK, Waheed A, Ben-Avram CM, Shively JE, Lusis AJ (1987): Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc. Natl. Acad. Sci. USA 84: 1157-1161.

Ramsoondar J, Christopherson RJ, Guilbert LJ, Wegmann T (1993): A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biol. Reprod. 49: 681-694.

Rettenmier CW, Roussel MF, Ashmun RA, Ralph P, Price K, Sherr CJ (1987): Synthesis of membrane-bound colony-stimulating factor-1 (CSF-1) and down-modulation of CSF-1 receptors in NIH 3T3 cells transformed by cotransfection of the human CSF-1 and c-fms (CSF-1 receptor) genes. Mol. Cell Biol. 7: 2378-2387.

Rothwell VM, Rohrschneider LR (1987): Murine c-fms cDNA: cloning, sequence analysis and retroviral expression. Oncogene Res. 1 (4), 311-324.

Samuel CA, Perry JS (1972): The ultrastructure of pig trophoblast rransplanted to an ectopic site in the uterine wall. J. Anatomy 113(1):139-149.

Samuel CA (1972): Ultra-structural features of the ectopic pig trophoblast. J. Physiol. 226(2): 22P.

Samuel CA (1971): The development of pig trophoblast in ectopic sites. J. Reprod. & Fertil. 27(3): 494-495.

Sapi E. Kacinski BM. The role of CSF-1 in normal and neoplastic breast physiology. Proceedings of the Society for Experimental Biology & Medicine. 220(1):1-8, 1999

Sapi E, Flick MB, Rodov S, Glimore-Hebert M, Kelley M, Rockwell S, Kacinski BM (1996): Independent regulation of invasive and anchorage-independent growth by different autophosphorylation sites of the macrophage colony-stimulating factor 1 receptor. Cancer Res. 56: 5704-5712.

Seelentag WK, Mermod JJ, Montesano R, Vassalli P (1987): Additive effects of interleukin-1 and tumour necrosis factor-alpha on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. EMBO J. 6(8): 2261-2265.

Sherr CJ (1990): Colony-stimulating factor-1 receptor. Blood 75(1):1-12.

Sherr CJ, Stanley ER (1990): Colony stimulating factor-1 (macrophage colony stimulating factor). In Sporn MB, Roberts AB (ed): "Peptide Growth Factors and Their Receptors (Vol.1)". New York: Springer-Verlag, pp 667-698.

Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER (1985): The c-fms protooncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41(3): 665-676.

Stanley ER, Guilbert LJ, Tushinski RJ, Barteimez SH (1983): CSF-1: a mononuclear phagocyte lineage-specific growth factor. J. Cell Biochem. 21: 151-159.

Stanley ER (1990): Role of colony stimulating factor-1 in monocytopoiesis and placental development. In Mahowald A (ed.): "Genetics of Pattern Formation and Growth Control". New York: Wiley-Liss, pp 165-180.

Tuo W, Harney JP, Bazer FW (1995): Colony-stimulating factor-1 in conceptus and uterine tissues in pigs. Biol. Reprod. 53: 133-142.

Visvader J, Verma IM (1989): Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. Mol & Cellular Biol. 9(3):1336-1341.

Wong GG, Temple PA, Leary AC, Witek-Giannotti JS, Yang YC, Ciarletta AB, Chung M, Murtha P, Kriz R, Kaufman RJ, Ferenz CR, Sibley BS, Turner KJ, Hewick RM, Clark SC, Yanai N, Yokota H, Yamada M, Saito M, Motoyoshi K, Takaku F (1987): Human CSF-1: molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. Science 235: 1504-1508.

Wood GW, Hausmann E, Choudhuri R (1997): Relative role of CSF-1, MCP-1/JE, and RANTES in Macrophage Recruitment During Successful Pregnancy. Mol. Reprod. Dev. 46: 62-70.

Yelich JV, Pomp D, Geisert RD (1997): Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biol. Reprod. 57(5): 1256-1265.

Yen A, Sturgill R, Varvayanis S (1997): Increasing c-FMS (CSF-1 receptor) expression decreases retinoic acid concentration needed to cause cell differentiation and retinoblastoma protein hypophosphorylation. Cancer Res. 57(10): 2020-2028.

CHAPTER 3

EXPRESSION OF MATRIX METALLOPROTEINASES AND THEIR REGULATORS DURING PORCINE PERI-IMPLANTATION DEVELOPMENT

INTRODUCTION

Unlike rodent and human blastocysts that start to attach to, and penetrate into, the endometrium immediately after hatching, porcine blastocysts undergo a prolonged pre-implantation development period involving an initial expansion, followed by appropriate spacing and conceptus elongation and subsequent attachment in the uterus (Flood, 1991). Upon expanding to a diameter of about 10 mm, porcine blastocysts elongate dramatically from a spherical to a filamentous form that can be up to 100 cm in length. This elongation precedes the physical attachment which occurs between the trophoblast and the uterine epithelium (Geisert et al., 1982a). During initial elongation from Day 10 to 12 of pregnancy (Day O designated as onset of estrus), the physiological activities of the blastocyst increase, as evidenced by the onset of estrogen (Gadsby et al., 1980; Fischer et al., 1985) and protein synthesis (Godkin et al., 1982). The onset of embryonic estrogen synthesis is followed by a significant increase in intraluminal calcium content and a rapid release of secretory vesicles from uterine epithelial cells into the lumen (Geisert et al., 1982b; Stroband & Van der Lende, 1990). Along with the elongation process, the extra-embryonic mesoderm initiates development and migrates between the trophoblast and the extraembryonic endoderm (Patten, 1948). The trophoblast and the uterine epithelium become closely apposed to each other immediately after the blastocyst elongation is complete (Flood, 1991). This is followed by adhesion between these two cell layers around Day 14 of pregnancy, as seen by microvillar

interdigitation between the two epithelial layers by Day 16 of pregnancy (Dantzer, 1985; Keys and King, 1990).

Implantation of the blastocyst is an essential step during mammalian pregnancy, leading to formation of a placenta capable of supporting embryonic/fetal development to term. The endocrine milieu of the mammal, and in particular its progesterone and estrogen status, is essential for establishing appropriate uterine conditions for implantation (de Ziegler et al., 1998; Ace and Okulicz, 1995; Bazer et al., 1982). The processes leading to early embryonic development and implantation also involve non-hormonal factors, which include the expression of a variety of adhesion molecules, vasoactive agents, tissue remodeling enzymes and their regulators, as well as cytokines/ growth factors. Matrix metalloproteinases (MMP) or matrixins are a group of at least 16 Zn^{2+} endopeptidases, which can degrade extracellular matrix (ECM) components such as collagen(s), laminin, fibronectin and vitronectin (Parsons et al. 1997; Nagase, 1997; Benbow and Brinckerhoff, 1997). Most of the MMPs are secreted as zymogens (proMMPs), and as an example activation of proMMP-2 at the cell surface can be mediated *in vivo* by membrane type-1 MMP (MMP-14 or Mt1-MMP) that is abundantly expressed in placenta (Parsons et al., 1997; Corcoran et al., 1996; Will et al., 1995). Zymogens of other MMPs can be activated through the action of plasmin by urokinase-type plasminogen activator (uPA) (Parsons et al., 1997). MMPs play a major role in tissue remodeling in a variety of physiological processes, which include embryo development, morphogenesis, angiogenesis and tissue involution, as well as pathological processes such as tissue ulceration, arthritis and cancers (Damsky et al., 1997; Woessner, 1994). MMPs have also been implicated in the subtle modulation of cell-matrix

interactions governing processes as diverse as cellular differentiation and migration (Basbaum and Werb, 1996). Tissue inhibitors of metalloproteinases (TIMPs) are the specific inhibitors for MMPs, and there are four members characterized in this family. TIMP-1 and TIMP-2 are present in a soluble form and play a key role in maintaining the balance between ECM deposition and degradation in different physiological processes (Gomez et al., 1997). TIMP-3 is insoluble, and is the only member of the TIMP family that is found exclusively in the ECM (Leco et al., 1994). TIMP-4 is mainly expressed in the adult heart with low levels in the placenta in the human, and is mainly expressed in adult brain and heart in the mouse (Leco et al., 1997; Greene et al., 1996). The role of MMPs and TIMPs during embryo implantation has been extensively studied in the mouse and human (Damsky et al., 1997; Bass et al., 1997; Alexander et al., 1996). In the pig, a previous study on the localization and expression of MMPs and TIMPs at the time of embryo-uterine contact was carried out by Menino et al. (1997).

Embryo implantation during pregnancy in pigs is a non-invasive process that results in simple attachment between the trophoblast and the uterine epithelium. This type of placentation is termed epitheliochorial to distinguish it from other more invasive types of placentation, such as the hemochorial placentation seen in rodents and humans. Strikingly, when porcine embryos were artificially transplanted to ectopic sites, the trophoblast cells appeared to be invasive (Samuel, 1971and 1972; Samuel and Perry, 1972). This suggests that either the environmental cues present at the ectopic site caused these cells to become invasive or, alternatively, that the intrinsically invasive nature of these cells must be regulated *in vivo* during normal conceptus development. If the latter is correct, then the balance between ECM degradation and deposition in the embryo, in the

endometrium, and at the interface between the trophoblast and the uterine epithelium must be tightly controlled, during the peri-implantation period, such that implantation and early embryogenesis can proceed appropriately. The objective of this study was to characterize the temporal expression of MMPs and their regulators in blastocysts and the endometrium during the peri-implantation period in the pig.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, with two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 00:00) for onset of standing heat with a vasectomized boar. At 16 and 24 hours after onset of standing heat, gilts were artificially inseminated using fresh-pooled crossbred semen. In an initial series of animals, pregnant gilts were slaughtered on Days 9.5, 11, 11.75, 12, 13.5, 15.5, 15.75 and 15.75 of pregnancy, respectively (Day 0 defined as onset of standing heat). For each of these gilts, jugular blood samples were taken every 4h from the eighteenth day of the previous estrous cycle until the fifth day after onset of estrus. Plasma luteinizing hormone (LH) concentrations were quantified by radioimmunoassay as described by De Rensis et al. (1993). Based on the time of the preovulatory LH surge and the predicted time of ovulation, a revised estimate of day of pregnancy was made (Fig. 3.1 and Table 3.1).

Since a limited amount of RNA samples could be extracted from blastocysts at these early developmental stages, a secondary series of animals were slaughtered on Days 10.5, 11, 12, 14, 16 of pregnancy (Day 0 defined as onset of standing heat), respectively,

to obtain sufficient blastocyst materials for completing the analysis of gene expression in this experiment. In addition, the uterine fluids of pregnant gilts obtained from Day 3, 5.5, 7, 9, 10.5, 12 and 14 of pregnancy (Day 0 defined as onset of standing heat) were used for examination of proMMP-2, proMMP-9 and TIMPs levels.

B. Collection of Cells, Blastocysts and Endometrial tissues

The reproductive tract was recovered within 10 minutes of slaughter. Uterine horns were excised and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings, pooled within individual pigs, and rinsed briefly in physiological saline to remove uterine debris prior to snap-freezing in liquid nitrogen.

Sections of endometrial tissues were harvested from the mid-section of each uterine horn of the initial series of pregnant gilts. The endometrial tissues were immediately snap-frozen in liquid nitrogen after collection. All samples were stored at – 80 °C.

A porcine trophoblast cell line, Jag-1, was included for comparison of gene expression, and the cultured trophoblast cells were harvested before they reached confluence, as described previously (Ramsoondar et al., 1994).

C. RNA Extraction

Total cellular RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues or cultured cells, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen prior to addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

D. Primers and RT-PCR

All PCR primer sequences and the expected product sizes are listed in Table 3.2. Design of PCR primers for MMP-1, MMP-2, MMP-8, MMP-11, MMP-13, MMP-14, TIMP-1 and TIMP2 were based on the conserved region of human and mouse cDNA sequences. MMP-9, TIMP3 and uPA primers were synthesized according to Menino et al. (1997). and primers for G3PDH and β-actin were designed according to Yelich et al. (1997).

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 μg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 μl, containing 5 μM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% Tween 20 (v/v). The RT reaction could be scaled up as needed and the final reaction mixture was stored at 4°C prior to use. Aliquots from the same RT reaction were used to amplify each of the target genes including G3PDH (Table 3.3) for each RNA sample, and one reaction mixture was prepared for PCR amplification of all target genes in that sample. Therefore, the PCR amplification of all target genes for each sample was carried out under the same conditions, except that the amount of cDNA templates, the annealing temperature and the cycle number of PCR were optimized empirically to each of these target genes (Table 3.2). All PCR reactions were carried out in a Perkin Elmer GenAmp 9600 Thermocycler in a reaction volume of 50 μl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 μM of each specific primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. The identities of the amplified

products were confirmed by sequencing and/or diagnostic restriction enzyme analysis. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes.

E. Substrate Gel Electrophoresis (Zymography and Reverse Zymography)

Uterine flushings were collected and centrifuged at 1,500 xg for 10 min at 4°C to remove tissue debris. Total protein was quantified using the BCA protein assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Zymography was carried out as described by Lantz and Ciborowski (1994), and reverse zymography was performed as described by Staskus et al. (1991). Briefly, samples balanced for protein content were mixed with SDS loading buffer, and loaded on 15 % (w/v) SDS-polyacrylamide gel containing 1 mg/ml gelatin (Type A) (Sigma, Mississauga, ON, Canada). After separation by electrophoresis at 4 °C, enzymes were renatured by incubating the gel once in 2.5% (w/v) Triton X-100 (Sigma, St. Louis, MO, USA) for 30 min. The gel was then washed twice in 50 mM Tris-HCl pH 7.4 for 15 min, which was followed by incubation for 26 hr at 37 °C in gelatinolysis buffer (50 mM Tris-HCl pH 7.4 containing 10 mM $CaCl_2$). The gel was stained with Amido Black. Degradation of gelatin in the gel due to the action of proteinases was revealed as transparent bands. For reverse zymography, a crude mixture of gelatinases from serum-free conditioned medium of HT1080 cells (Giambernardi et al., 1998) was incorporated into 20% (w/v) polyacrylamide gel containing gelatin. Electrophoresis was carried out exactly as described above for zymography. Inhibitor activities were revealed as dark bands against the partially clear background.

RESULTS

A. Assessment of Developmental Stages of Conceptuses

Most of the studies of reproduction in pigs have designated onset of estrus (standing heat) as Day 0 of pregnancy. Recent data have confirmed that onset of estrus is not an accurate predictor of the time of ovulation since ovulation can occur from 10 to 85 h after onset of estrus in sows and from 23 to 48 h in gilts (Soede and Kemp, 1997). The average duration between the onset of estrus and ovulation is 44 hr with a range between 30 to 60 h based on an ultrasound study of 90 gilts used in our laboratory (Alemeida et al., 1999 manuscript under review). Therefore, the developmental stages of conceptuses from individual gilts used in our laboratory could vary by as much as 30 h if they are estimated by onset of estrus.

In order to assess developmental stages of conceptuses more accurately, the peak of the preovulatory LH surge was estimated for the initial series of gilts in the present study by measuring the LH concentration in the jugular blood during the peri-ovulation period. It is known that ovulation occurs approximately 30-35 h after the peak of the preovulatory LH surge (Kemp and Soede, 1997; Soede et al., 1994). Therefore, Day 0 of pregnancy is defined in the present study as the period between 14 hr before and 10 hr after the peak of LH surge (Fig. 3.3). In this way, the variation in the developmental stage estimated for conceptuses from individual gilts could be theoretically reduced from 30 h to 4.5 h (2 h from the estimation of the peak of LH surge plus 2.5 h from the interval between the ovulation and the peak of LH surge). As a result, the developmental stages of conceptuses could be defined more precisely. Despite this, the developmental stages of

conceptuses from the initial series of gilts in the present study followed the same temporal order whether they were determined by onset of estrus or by the peak of the LH surge, with the exception of the two gilts No. 170 and 367 (Table 3.1). Since the LH data for the secondary series of gilts in the present study were not available, the developmental stages for the conceptuses from these gilts were based on the onset of estrus. Where possible, the data is presented in terms of both methods of estimation.

B. Expression of MMP-2 and MMP-9

Transcripts of G3PDH were expressed at relatively constant levels in both endometrial tissues and blastocysts (Fig. 3.2F, 3.3F & 3.3N). Expression of MMP-2 (gelatinase A) transcripts was variable in the endometrium (Fig. 3.2G), whereas MMP-2 transcripts increased gradually in blastocysts obtained between Days 11 and 15 of pregnancy (Fig. 3.3G). ProMMP-2 was detected by zymography in uterine flushings of gilts obtained between Day 3 and 14 of pregnancy, with elevated levels on Day 3 and Day 14, respectively (Fig. 3.4A&3.4C). MMP-9 (gelatinase B) transcripts were not detectable in the endometrium unless a higher amount of cDNA template (10 μl) and a higher number of PCR cycles (45) was used for amplification (Fig. 3.2C). Under these conditions, transcripts of MMP-9 were still not detectable in any blastocysts examined, although they were revealed at low levels in Jag-1 cells (Fig. 3.3C). ProMMP-9 was only detected in uterine flushings obtained on Day 3 and 7 of pregnancy, respectively, and not on Days 9, 10.5, 12, 14 of pregnancy (Fig. 3.4A).

C. Expression of MMP-14, uPA and TIMPs

MMP-14 transcripts were constantly detected at high levels in endometrial tissues obtained between Day 8.7 and 15 of pregnancy (Fig. 3.2K). MMP-14 transcripts

were observed at low levels in blastocysts of Day 11 but increased dramatically in blastocysts obtained between Day 12 and 15 (Fig 3.3K). Expression of uPA transcripts slightly decreased in endometrial tissues collected between Day 9 and 15.75 of pregnancy (Fig. 3.2M), whereas it exhibited a biphasic pattern in blastocysts obtained between Day 11 and 15, with peak levels on Day 11.2 and Day 15, respectively (Fig. 3.3M). In endometrial tissues obtained between Day 9 and 15.75 of pregnancy, TIMP-1 and -3 transcripts were detected at variable levels, while TIMP-2 transcripts increased slightly (Fig. 3.2D, 3.2E & 3.2L). Transcripts of TIMP-2 were not detected in blastocysts obtained on Day 11 (gilt No. 399), but increased in blastocysts obtained between Day 11 (gilt No. 153) and Day 15 of pregnancy (Fig. 3.3L). Levels of TIMP-1 and TIMP-3 transcripts were lower in blastocysts obtained on Day 11, and dramatically increased in blastocysts collected on Day 12, 14 and 16 (Fig. 3.3D & 3.3E). TIMP-2 activity was not detected by reverse zymography in uterine flushings of pregnant gilts collected on Day 3, 5.5 and 7 of pregnancy, but was observed in uterine flushings obtained between Days 9 to 14 of pregnancy, with higher levels on Day 14 (Fig. 3.4B&3.4C).

D. Expression of Other MMP Transcripts

Expression of three types of interstitial collagenases, MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3) was observed during the peri-implantation period. MMP-1 transcripts were only detected at low levels in endometrial tissues collected on Day 12.2 and 15 of pregnancy (Fig. 3.2A). Expression of MMP-1 transcripts was not detected in blastocysts obtained on Day 10.5, 11 and 12, although it was observed at low levels in blastocysts obtained on Day 14 and 16 (Fig. 3.3A). In endometrial tissues, expression of MMP-8 transcripts was not constant and was high on

Day 8.7, 11 (gilt No. 399), 11.2 and 12.2 of pregnancy and low on Day 14.5 of pregnancy (Fig. 3.2B). In contrast, expression of MMP-8 transcripts was up regulated gradually in blastocysts obtained between Days 10.5 and 16 of pregnancy (Fig. 3.3B). MMP-13 transcripts were not detected in any endometrial tissues (Fig. 3.2J). However, MMP-13 transcripts were detected at low levels in blastocysts obtained on Day 14.5 of pregnancy but not in blastocysts obtained from other developmental stages (Fig. 3.3J).

MMP-11 transcripts were observed at low levels in endometrial tissues obtained on Day 14, 14.5 and 15 of pregnancy but not in endometrial tissues collected from other developmental stages (Fig. 3.2I). However, MMP-11 transcripts were not detected in blastocysts from all developmental stages examined (Fig. 3.3I). Expression of MMP-7 (matrilysin) transcripts was not detected in any endometrial tissues examined (Fig. 3.2H). In contrast, MMP-7 transcripts were detected in blastocysts obtained on Day 15 but not in blastocysts collected at other developmental stages (Fig. 3.3H).

DISCUSSION

Convincing evidence demonstrates that the invasive properties of mouse and human trophoblast cells are due to the secretion of matrix metalloproteinases (MMP), especially MMP-9, that can degrade ECM components (Morgan et al., 1998; Bass et al., 1997; Alexander et al., 1996; Hurskainen et al., 1996). In the mouse, it has been shown that MMP-9 is the predominant metalloproteinase expressed by trophoblast cells of the implanting embryo and TIMP-3 is the major metalloproteinase inhibitor in the maternal decidua. During mouse embryo implantation, MMP-2, TIMP-1 and TIMP-2 were expressed mainly in the undifferentiated stroma outside the decidua, and TIMP-3 was

expressed in primary and mature decidual cells during their differentiation (Alexander et al., 1996). Expression of MMP-9 was not detected in uterine stromal cells except of those at the site of implantation. It has also been shown that MMP-9 activity is a rate-limiting factor for mouse trophoblast cells to invade and degrade ECM *in vitro* (Behrendtsen et al. 1992). Therefore, pronounced expression of MMP-9 in trophoblast giant cells, coupled with high-level expression of TIMP-3 in the stroma surrounding the embryo, contributes to the regulated invasive implantation in the murine uterus (Das et al., 1997; Leco et al., 1996; Reponen et al., 1995; Harvey et al., 1995). Similarly, expression of both MMP-9 and TIMP-3 is up-regulated in human cytotrophoblasts along the invasive pathway, which plays an important role in regulating the depth of blastocyst invasion in the human uterus (Bass et al., 1997; Shimonovitz et al., 1994; McMaster et al., 1994; Librach et al., 1991). Furthermore, MMP-9 has been shown to be the crucial factor for human trophoblast cells to invade ECM *in vitro* (Morgan et al., 1998). Therefore, MMP-9 appears to be a common effector for trophoblast invasion during embryo implantation in mice and humans (Salamonsen, 1999).

During normal embryo implantation in the pig, trophoblast cells of blastocysts do not penetrate the basement membrane of the endometrium. In the present study, MMP-9 transcripts were not detected in blastocysts although they were weakly expressed in the endometrium during the peri-implantation period and in trophoblast cells cultured *in vitro*. This result is in agreement with a previous study in which MMP-9 transcripts were not detected in porcine blastocysts and the endometrium at the time of embryo-uterine contact by RT-PCR and *in situ* hybridization, although they could be detected by Southern blot analysis of RT-PCR products (Menino et al., 1997). These earlier results

were extended in the present study by zymography data showing that MMP-9 activity was not detected in uterine fluids representing Day 9 to 14 of pregnancy. In contrast, expression of TIMP-1, TIMP-2 and TIMP–3 transcripts was observed in both blastocysts and the endometrium, and TIMP-2 activity was also detected in uterine fluids from gilts representing Day 9 to 14 of pregnancy. Taken together, these results suggest that little or no production of MMP-9, coupled with high levels of TIMP-1, TIMP-2 and TIMP–3 expression in blastocysts during embryo attachment, could be responsible for the non-invasive type of implantation seen in pigs. These results are in agreement with previous studies on the role of MMP-9 in regulating embryo implantation in mice and humans with invasive hemochorial placentation.

MMP-9 transcripts and active MMP-9 enzyme were not detected in blastocysts and uterine fluids, respectively, at the time of embryo-uterus contact by the very sensitive approaches used in the present study. Therefore, the trophoblast cells of implanting porcine blastocysts do not appear to produce significant levels of MMP-9 during normal pregnancy. In contrast, low levels of MMP-9 transcripts were observed in Jag-1 trophoblast cells in the present study, and significant levels of proMMP-9 activity was also observed in conditioned media from Jag-1 cells (data not shown). In addition, the Jag-1 cell line was able to invade matrigel (artificial basement membrane) *in vitro* in our laboratory (Chai et al., unpublished results). These data suggest that porcine trophoblast cells are invasive *in vitro*, which provides further evidence at the molecular level for previous histological observations in which porcine trophoblast cells appear to be invasive after porcine conceptuses are transferred to ectopic sites (Samuel, 1971and 1972; Samuel and Perry, 1972). The differential behaviors of porcine trophoblast cells

under *in vivo* and *in vitro* conditions suggest that there might be some negative regulatory mechanisms, within the blastocsyt or in the uterine fluid, limiting the production of MMP-9 in trophoblast cells *in vivo*, possibly at the transcriptional level.

In addition to embryo implantation, tissue remodeling involving the actions of MMPs is a component of several other processes occurring during mammalian embryogenesis, including cell migration, cell to cell interactions, and embryo expansion (Brenner et al., 1989). It has been shown that metalloproteinases have distinct roles in the differentiation and migration of murine parietal endoderm, the first embryonic migratory cells that adhere to ECM secreted from trophoblast cells (Behrendtsen and Werb, 1997). MMP-2 is secreted as a proenzyme that can form a specific stoichiometric complex with TIMP-2. Activation of the secreted proenzymes and interaction with TIMP-2 determine the net enzymatic activity in the extracellular space. The cell-surface activation of proMMP-2, involving the formation of a tri-molecular complex of MMP-14.MMP-2.TIMP2, is considered to be critical for cell migration and invasion. Activated MMP-14 was shown to act as a cell surface receptor for TIMP-2, and this bi-molecular complex in turn acts as a receptor for MMP-2 to recruit proMMP-2 to the cell surface (Strongin et al., 1995). Moreover, TIMP-2 bound to the plasma membrane was shown to be the specific inhibitor of the cell surface-activated MMP-2 (Itoh et al., 1998; Strongin et al., 1993). Activation of MMP-2 on the cell surface provides a basic mechanism for spatially regulated extracellular proteolysis. More interestingly, a number of investigators have recently shown that MMP-2 is co-expressed temporally and spatially with its activator MMP-14 and its inhibitor TIMP-2 in mouse tissues during embryogenesis, and in human fetal membranes (Apte et al., 1997; Kinoh et al., 1996; Fortunato et al., 1998). In the

present study, expression of MMP-2, MMP-14 and TIMP-2 transcripts was up-regulated in embryonic tissues with the progression of embryonic development, and elevated levels of proMMP-2 and TIMP-2 were also observed in uterine fluids of pregnant gilts at the time of embryo-uterus contact. The co-expression of MMP-2, TIMP-2 and MMP-14 in porcine blastocysts during the peri-implantation period might contribute to the spatially regulated extracellular proteolysis required for the extraembryonic mesoderm migration from the primitive streak region in porcine blastocysts.

The levels of proMMP-2 were shown to be very high in uterine fluids of pregnant gilts obtained between Day 9 to 14 of pregnancy in the present study. Since MMP-2 transcripts were both expressed in endometrial tissues and blastocysts obtained between Day 11 to 15 of pregnancy, the high levels of proMMP-2 in the uterine fluid could be contributed by secretion from both implanting blastocysts and the endometrium. This enhanced secretory activity might be a response to the increased embryonic estrogen content known to exist in uterine fluids during this period (Gadsby et al., 1980; Geisert et al., 1982b; Fischer et al., 1985). Additional protein species with strong gelatinolytic activity were also detected by zymography of uterine flushings from a pregnant gilt obtained on Day 14 of pregnancy. These protein species might represent the active form (62 kDa) and low molecular mass forms of MMP-2, or proMMP-3, both of which also degrade gelatin (Salamonsen et al., 1995; Nagase, 1991).

Although expression of TIMP-1, TIMP-2 and TIMP-3 transcripts was detected in both blastocysts and the endometrium, strong activity of TIMP-2, but not TIMP-1 and TIMP–3, was observed in uterine fluids in the present study. These results are consistent with a previous study in which transcripts of TIMP-1 and TIMP-3 were mainly localized

in endodermal cells, whereas those of TIMP-2 were detected in all cells of the blastocyst (Menino et al., 1997). In addition, TIMP-3 is found exclusively in the ECM of a large number of cultured human cells but not in the conditioned media (Kishnani et al., 1995). High levels of TIMP-2 in the uterine fluid of pregnant gilts could play a major role in preventing potential damage to the integrity of the uterine epithelium by the large amount of proMMP-2, existing in the uterine lumen as shown in the present study, at the time of embryo-uterus contact. However, levels of TIMP-1, -2 and –3 transcripts were shown to be up-regulated during the peri-implantation period in the present study, which suggests that TIMP-1, -2 and –3 might all be involved in maintaining the balance between ECM deposition and degradation in blastocysts during the mesoderm outgrowth.

It is interesting to note that the expression of uPA transcripts appears to be biphasic in porcine blastocysts during the peri-implantation period examined. This extends a previous observation on uPA enzyme activities released by porcine blastocysts isolated from pregnant pigs representing the same stages of pregnancy (Fazleabas et al., 1983). Coincidentally, expression of uPA observed in the present study is temporally correlated with estrogen synthesis in porcine blastocysts that has been previously shown to peak around Day 11 and Day 16 of pregnancy, respectively (Pusateri et al., 1990, Geisert et al., 1987; Heap et al., 1981; Zavy et al., 1980). It is known that estrogen could down-regulate the expression of uPA mRNA in a dose-dependent fashion in human breast cancer cells (Leverson et al., 1998). Therefore, we hypothesize that embryonic estrogen might be responsible for dramatic changes in the production of uPA by blastocysts. Together with earlier *in situ* hybridization data showing that uPA transcripts were mainly localized to the extraembryonic endoderm with little expression in the

trophoblast cells (Menino et al., 1997), our results suggest that the proteolytic cascade driven by uPA through plasmin might be required for early porcine embryogenesis. The ultimate activity of uPA in the uterine lumen released from blastocysts could be regulated by inhibitors of both uPA and plasmin, existing in the uterine lumen as shown by Mullins et al. (1980) and Fazleabas et al. (1983).

Expression of MMP-1, MMP-8 and MMP-13 transcripts was observed in porcine blastocysts in the present study, which suggests that they might be involved in the tissue remodeling occurring during porcine embryogenesis. Low levels of MMP-1 transcripts were detected in endometrial tissues of pregnant gilts obtained at the time of embryo-uterine contact in the present study, which is consistent with a previous observation in uterine tissues of pregnant ewes (Hampton et al., 1995). MMP-8 (collagenase-2) is also known as neutrophil collagenase because of its exclusive expression in the neutrophil under inflammatory conditions (Jeffrey, 1998). The present study showed that MMP-8 transcripts were expressed at variable levels in the endometrium, suggesting that neutrophils might be recruited to the endometrium in response to the implanting blastocysts. However, expression of MMP-8 transcripts is found to increase gradually in porcine blastocysts during the peri-implantation in the present study, which correlates with the formation of extraembryonic mesoderm. These results are in agreement with the observation of MMP-8 expression at late stages during mouse embryogenesis, coinciding with the appearance of hematopoietic cells, and also in the *post partum* uterus (Balbin et al., 1998). MMP-13 transcripts were not detected in the endometrium of pregnant pigs in the present study, which extends a previous observation in the mouse uterus (Das et al., 1997). MMP-7 was first discovered in the involuting rat

uterus and has been termed the uterine metalloproteinase. It is capable of degrading a wide range of ECM components and can activate several other MMPs including collagenases (Woessner, 1996; Wolf et al., 1996). However, MMP-7 transcripts were not observed in porcine endometrial tissues during the stages of pregnancy examined, were observed in Jag-1 cells, and were strongly expressed in blastocysts at the time of embryo-uterine contact in the present study. These data suggest that MMP-7 might be involved in the activation of proMMP-2 in porcine blastocysts as suggested in the activation of human proMMP-2 by Crabbe et al. (1994).

Previous studies on gene expression during porcine embryo development have been carried out with reference to onset of standing heat. Recently, Soede and Kemp (1997) reported that the onset of standing heat was not a good predictor of the time of ovulation. Therefore, particularly in early gestation, determination of developmental stages of porcine embryos based on the onset of standing heat might not be very accurate. Based on known endocrine relationships, the preovulatory LH surge can be used as a more accurate predictor of the time of ovulation than the onset of standing heat, and therefore of true developmental age. As shown in the present study (Table 3.1), developmental stages of embryos isolated from pregnant gilts No. 399 and No. 153 were Days 11 and 11.75, respectively, based on the onset of standing heat, but they were both Day 11 according to the time of preovulatory LH surge. In contrast, developmental stages of embryos isolated from pregnant gilts No. 170 and No. 367 were both Day 15.75 based on the onset of standing heat, but they were Days 14.5 and 15.0, respectively, according to the time of preovulatory LH surge. In future studies that examine the gene expression during early embryo development, we believe that accurate assessment of the

developmental stages of embryos and/or conceptuses is needed. In addition, it should be noted that the number of animals used in the present study is limited, although the number of blastocysts is reasonably sufficient for representing individual developmental stages of embryos.

In summary, we have examined the temporal expression of MMPs and their regulators in the endometrium and blastocysts during the peri-implantation period. Expression of these protein factors correlate with conceptus implantation and early embryogenesis in the pig, which are the two major biological processes coordinated during this period. Little or no expression of MMP-9 in blastocysts during the peri-implantation period may be the basis for the non-invasive implantation/ attachment process seen in the pig. The co-expression of MMP-2, TIMP-2 and MMP-14 might contribute to the mesoderm migration during early embryogenesis in the pig.

Table 3.1 Determination of developmental stages of pregnant gilts based on the onset of estrus and the peak of the preovulatory LH surge, respectively

Pig No.	Day of pregnancy (based on onset of estrus)	Date and time of the peak of the LH surge	Date and time of slaughter	Interval between LH peak and slaughter (h)	Day of pregnancy (based the LH data)
179	9.50	May 6; 8:00	May 15; 10:00	16+8x24+10=218	(218-10)/24=8.7
399	11.00	Aug 18; 24:00	Aug 30; 10:00	11x24+10=274	(274-10)/24=11.0
153	11.75	April 26; 24:00	May 8; 10:00	11x24+10=274	(274-10)/24=11.0
354	12.00	Aug 18; 20:00	Aug 30; 10:00	4+11x24+10=278	(278-10)/24=11.2
172	13.50	May 2; 20:00	May 15; 10:00	4+12x24+10=302	(302-10)/24=12.2
387	15.50	Aug 20; 24:00	Sept 4; 10:00	14x24+10=346	(346-10)/24=14.0
170	15.75	April 29; 24:00	May 15; 10:00	15x24+10=370	(370-10)/24=15.0
367	15.75	Aug 20; 12:00	Sept 4; 10:00	12+14x24+10=358	(358-10)/24=14.5

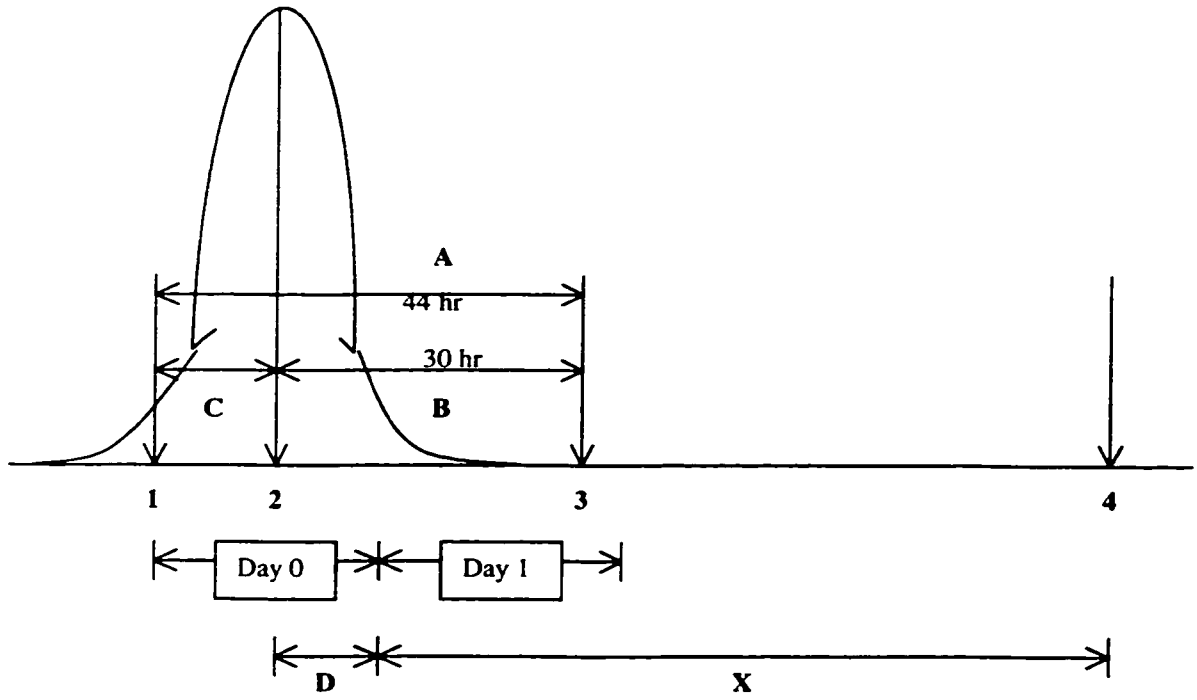
Table 3.2 PCR primers for target genes and their sequences

Target Genes	Primer sequences#	Predicted size (bp)	Source*
MMP-1	GCAGCCCAGATGTGGGGTGCCCG ACACTTCTGGGGTTTGGGGGCCG	571	
MMP-2	CTTCCCCCGCCAGCCCAAGTGGG GGTGAACAGGGCTTCATGGGGGC	510	
MMP-7	CCCAAAGAATGGCCAAGTTC TGCAGAAGCCCAGATGTGGA	420	
MMP-8	GATGGACCCAATGGAATCCTTGC TCATAGCCACTCAGAGCCCA	550	
MMP-9	ACGTGGACATCTTCGACGC CGAACCTCCAGAAGCTCTGC	357	Menino et al., 1997
MMP-10	GCTCTTCAGTGTGTGTGTCACC GGAAATGAAGTCCAAGCAGG	323	
MMP-11	GCTCTTCTTCTTGGCAGGC AACGCCAATAGTCTCCACCTCG	313	
MMP-13	TCTGGTCTGCTGGCTCACGC TAGGCAGCATCAATACGGTTGG	472	
MMP-14 (MT1-MMP)	ACCATGAAGGCTATGAGGCGCCC GTTGATGGATGCAGGCAGGCCCC	860	
uPA	GTCTGGTGAATCGAACTGTGGC GGCTGCAAACCAAGGCTG	538	Menino et al., 1997
TIMP-1	GTACCTGCGTCCCACCCCACC GGCAGGCAGGCCAGGTGGCGG	504	
TIMP-2	CCTCCTGCTGCTGGGGACGCTGC AGTCCTGGTGGCCTGCTTACGGG	643	
TIMP-3	GACCCCTTGGCTCGGGCTCATCG GCTGGTCCCACCTCTCCACGAAG	379	
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	452	Yelich et al., 1997
β-Actin	ATCTTGATCTTCATGGTGCTGGGC ACCACTGGCATTGTCATGGACTCT	545	Yelich et al., 1997

#All primer sequences were listed from 5' end to 3' end. * Primers were designed according to conserved regions of human and mouse cDNA sequences obtained from GenBank database unless indicated.

Table 3.3 PCR cycling conditions for amplification of target genes

Target genes	Annealing temp (°C)	No. of cycles	cDNA (µl)
G3PDH & β-Actin (Co-amplification)	60	35	1
G3PDH (Alone)	57	35	0.4
MMP-1	57	35	5
MMP-2	65	35	4
MMP-7	57	35	4
MMP-8	57	45	4
MMP-9	57	45	10
MMP-10	57	35	4
MMP-11	57	35	4
MMP-13	57	35	4
MMM-14 (Mt1-MMP)	57	35	4
uPA	57	35	5
TIMP-1	57	35	5
TIMP-2	65	35	4
TIMP-3	57	35	5



Point 1: Onset of estrus.
Point 2: The peak of LH surge.
Point 3: Ovulation.
Point 4: Slaughter
A: the average duration (44 hr) between the ovulation and observed onset of estrous behavior from 90 gilts.
B: the duration between points 2 and 3 (based on previous endocrine studies).
C: =A – B = 44 – 30 =14 hr
D: = 24 – 14 = 10 hr
X: Total days of pregnancy

Formula: X = (Interval duration between points 2 and 4 — D) / 24.

Figure 3.1 Diagram showing different points of time at which predicted onset of estrus and ovulation occur in relation to the peak of the LH surge.

Figure 3.2 RT-PCR amplification of MMP-1 (A), MMP-2 (G), MMP-7 (H), MMP-8 (B), MMP-9 (C), MMP-11 (I), MMP-13 (J), MMP-14 (K), uPA (M), TIMP-1 (D), TIMP-2 (L), TIMP-3 (E) and G3PDH (F) transcripts in the endometrium during the peri-implantation period. The same series of pregnant gilts were used for examination of all target genes, and their developmental stages were indicated above the gels. Days of pregnancy in top panel were determined based on the peak of the LH surge, and those in the bottom panel were determined relative to the onset of estrus. N, negative control. PCR was carried out with 35 cycles for each target gene in this experiment, except for amplification of MMP-8 and MMP-9 where 45 cycles applied. RT-PCR products were analyzed in 1.2 % agarose gel stained with ethidium bromide. The sizes of PCR products for target genes were indicated on the left side of gels. G3PDH was amplified as an internal control for gene expression.

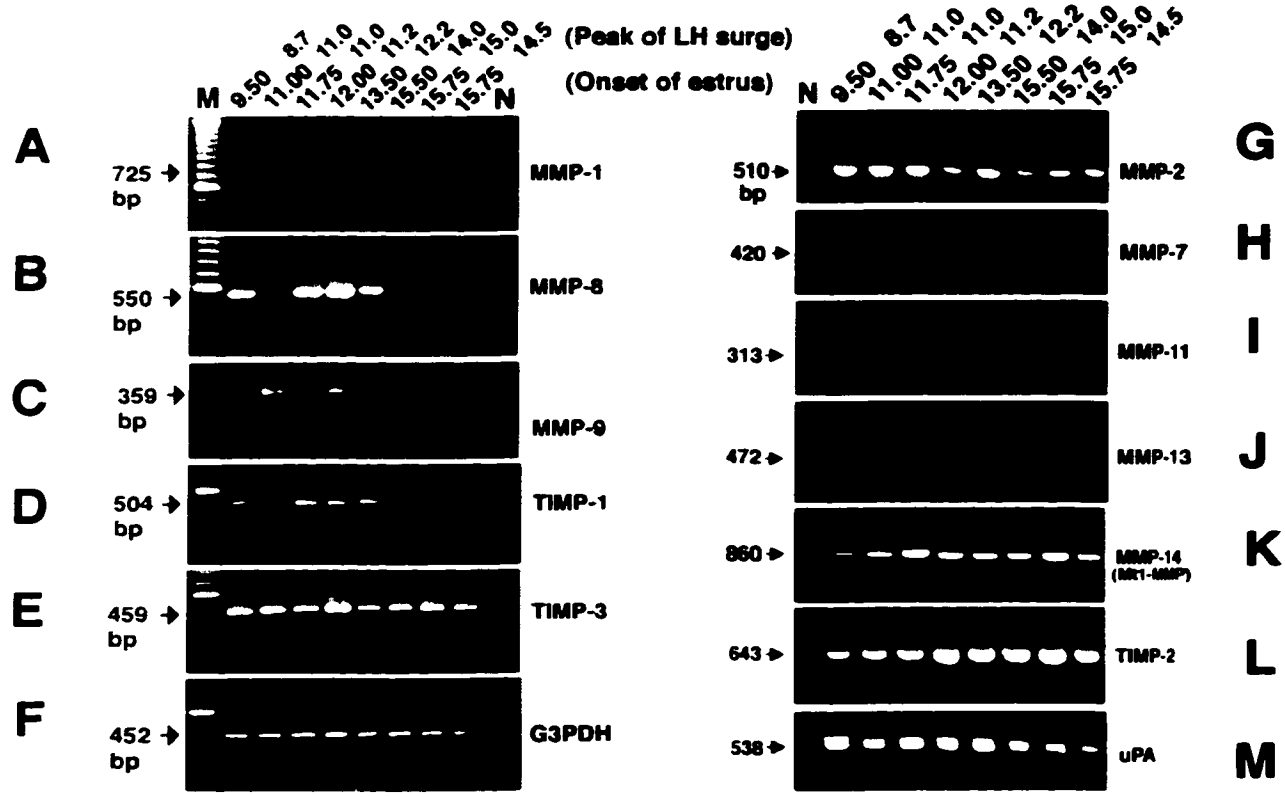

(Peak of LH surge)
(Onset of estrus)
M
N
9.50
11.00
11.75
12.00
13.50
15.50
15.75
8.7
11.0
11.2
12.2
14.0
15.0
14.5
A
B
C
D
E
F
G
H
I
J
K
L
M
725 bp
550 bp
359 bp
504 bp
459 bp
452 bp
510 bp
420
313
472
860
643
538
MMP-1
MMP-8
MMP-9
TIMP-1
TIMP-3
G3PDH
MMP-2
MMP-7
MMP-11
MMP-13
MMP-14
(MT1-MMP)
TIMP-2
uPA

Figure 3.3 RT-PCR amplification of MMP-1 (A), MMP-2 (G), MMP-7 (H), MMP-8 (B), MMP-9 (C), MMP-11 (I), MMP-13 (J), MMP-14 (K), uPA (M), TIMP-1 (D), TIMP-2 (L), TIMP-3 (E), G3PDH (F) and G3PDH & beta-actin (N) transcripts in blastocysts during the peri-implantation period. Two different series of pregnant gilts were used in this experiment, and their developmental stages were indicated above the gels. Days of pregnancy in top panel were determined based on the peak of the LH surge, and those in the bottom panel were determined relative to the onset of estrus. T, Jag-1 cells; N, negative control. PCR was carried out with 35 cycles for each target gene in this experiment, except for amplification of MMP-8 and MMP-9 where 45 cycles were applied.. RT-PCR products were analyzed in 1.2 % agarose gel stained with ethidium bromide. The sizes of PCR products for target genes were indicated on the left side of gels. Beta actin and/or G3PDH were amplified as internal controls for gene expression.

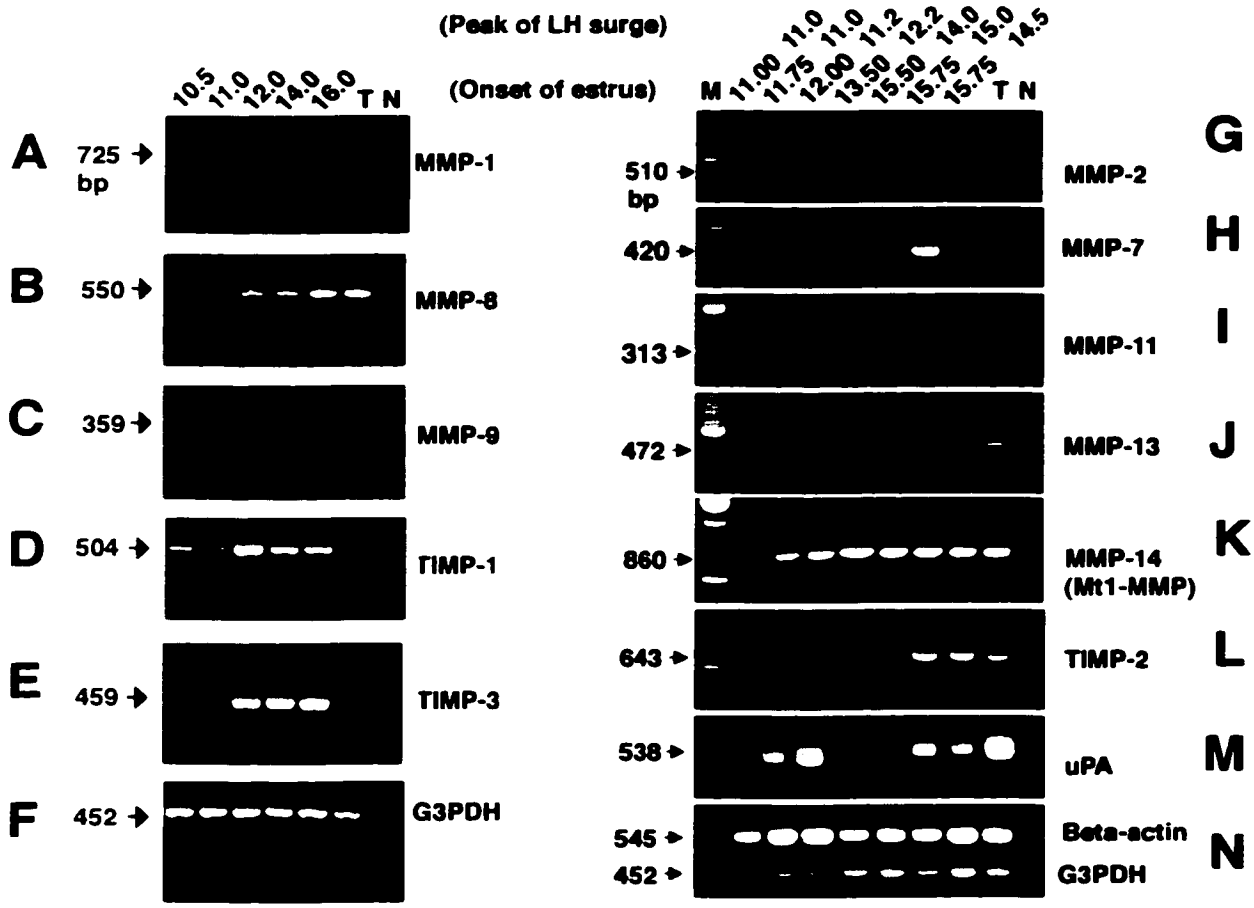
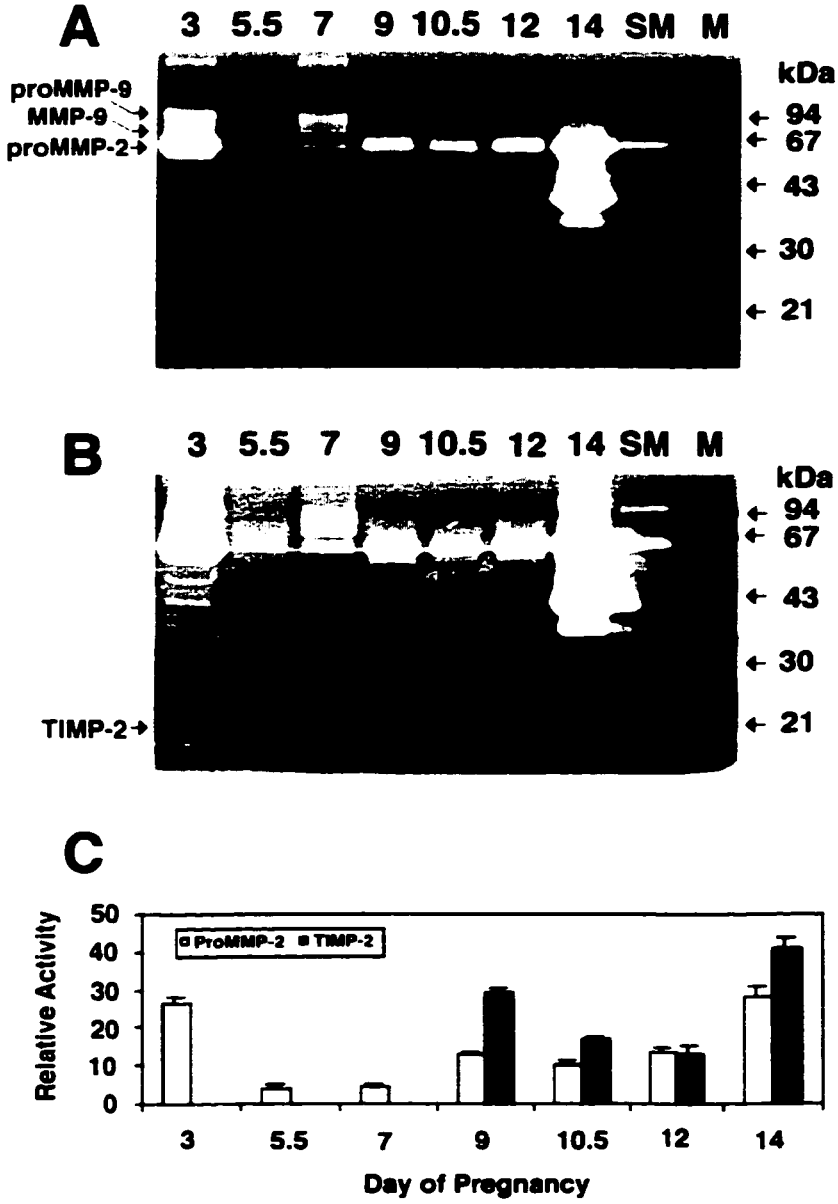

(Peak of LH surge)
(Onset of estrus)
10.5
11.0
12.0
14.0
16.0
T N
M
11.00
11.75
12.00
13.50
15.50
15.75
15.75
11.0
11.0
11.2
12.2
14.0
15.0
14.5
T N
A
725 bp
MMP-1
B
550
MMP-8
C
359
MMP-9
D
504
TIMP-1
E
459
TIMP-3
F
452
G3PDH
510 bp
MMP-2
G
420
MMP-7
H
313
MMP-11
I
472
MMP-13
J
860
MMP-14 (Mt1-MMP)
K
643
TIMP-2
L
538
uPA
M
545
Beta-actin
452
G3PDH
N

Figure 3.4 Gelatin zymographic (A) and reverse zymographic (B) analysis of MMPs and TIMPs in uterine flushings of gilts obtained on Days 3, 5.5, 7, 9, 10, 11 and 14 of pregnancy relative to onset of estrus, respectively. Total proteins of 10 μg were loaded on each lane. SM, total proteins of 20 μg from bovine skeletal muscle homogenate as positive controls for proMMP-2 and proMMP-9. M, molecular weight marker. (C) Relative levels of proMMP-2 and TIMP-2 in uterine fluids of pregnant pigs between Day 3 and 14 of pregnancy. Results were presented as the mean ± SEM of three separate experiments.


A
3 5.5 7 9 10.5 12 14 SM M
proMMP-9
MMP-9
proMMP-2
kDa
94
67
43
30
21
B
3 5.5 7 9 10.5 12 14 SM M
kDa
94
67
43
30
21
TIMP-2
C
ProMMP-2
TIMP-2
Relative Activity
50
40
30
20
10
0
3 5.5 7 9 10.5 12 14
Day of Pregnancy

REFERENCES

Ace CI. Okulicz WC. Differential gene regulation by estrogen and progesterone in the primate endometrium. Molecular & Cellular Endocrinology. 115(1):95-103, 1995

Alexander CM. Hansell EJ. Behrendtsen O. Flannery ML. Kishnani NS. Hawkes SP. Werb Z. Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. Development. 122(6):1723-36, 1996

Anderson LL. Growth, protein content and distribution of early pig embryos. Anatomical Record. 190(1):143-53, 1978

Apte SS. Fukai N. Beier DR. Olsen BR. The matrix metalloproteinase-14 (MMP-14) gene is structurally distinct from other MMP genes and is co-expressed with the TIMP-2 gene during mouse embryogenesis. Journal of Biological Chemistry. 272(41):25511-7, 1997

Balbin M. Fueyo A. Knauper V. Pendas AM. Lopez JM. Jimenez MG. Murphy G. Lopez-Otin C. Collagenase 2 (MMP-8) expression in murine tissue-remodeling processes. Analysis of its potential role in postpartum involution of the uterus. Journal of Biological Chemistry. 273(37):23959-68, 1998

Bass KE. Li H. Hawkes SP. Howard E. Bullen E. Vu TK. McMaster M. Janatpour M. Fisher SJ. Tissue inhibitor of metalloproteinase-3 expression is upregulated during human cytotrophoblast invasion in vitro. Developmental Genetics. 21(1):61-7, 1997

Bazer FW. Geisert RD. Thatcher WW. Roberts RM. The establishment and maintenance of pregnancy. In Control of Pig Reproduction, ed. by D.J.A. Cole and G.R. Foxcroft. Butterworths, London, UK. Pg: 227-252, 1982

Barends PM. Stroband HW. Taverne N. Kronnie G. Leen MP. Blommers PC. Integrity of the preimplantation pig blastocyst during expansion and loss of polar trophectoderm (Rauber cells) and the morphology of the embryoblast as an indicator for developmental stage. Journal of Reproduction & Fertility. 87(2):715-26, 1989

Basbaum CB. Werb Z. Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. Current Opinion in Cell Biology. 8(5):731-8, 1996

Behrendtsen O. Werb Z. Metalloproteinases regulate parietal endoderm differentiating and migrating in cultured mouse embryos. Developmental Dynamics. 208(2):255-65, 1997

Behrendtsen O. Alexander CM. Werb Z. Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development. 114(2):447-56, 1992

Benbow U. Brinckerhoff CE. The AP-1 site and MMP gene regulation: what is all the fuss about?. Matrix Biology. 15(8-9):519-26, 1997

Brenner CA. Adler RR. Rappolee DA. Pedersen RA. Werb Z . Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes & Development. 3(6):848-59, 1989

Corcoran ML. Hewitt RE. Kleiner DE Jr. Stetler-Stevenson WG. MMP-2: expression, activation and inhibition. Enzyme & Protein. 49(1-3):7-19, 1996

Crabbe T. Smith B. O'Connell J. Docherty A. Human progelatinase A can be activated by matrilysin. FEBS Letters. 345(1):14-6, 1994

Cross JC. Werb Z. Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science. 266(5190):1508-18, 1994

Damsky CH. Moursi A. Zhou Y. Fisher SJ. Globus RK. The solid state environment orchestrates embryonic development and tissue remodeling. Kidney International. 51(5):1427-33, 1997

Dantzer V. Electron microscopy of the initial stages of placentation in the pig. Anatomy & Embryology. 172(3):281-93, 1985

Das SK. Yano S. Wang J. Edwards DR. Nagase H. Dey SK. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period. Developmental Genetics. 21(1):44-54, 1997

De Rensis F. Cosgrove JR. Foxcroft GR. Luteinizing hormone and prolactin responses to naloxone vary with stage of lactation in the sow. Biology of Reproduction. 48 (5): 970-976, 1993

De Ziegler D. Fanchin R. de Moustier B. Bulletti C. The hormonal control of endometrial receptivity: estrogen (E2) and progesterone. Journal of Reproductive Immunology. 39(1-2):149-66, 1998

Fazleabas AT. Geisert RD. Bazer FW. Roberts RM. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. Biology of Reproduction. 29(1):225-38, 1983

Fischer HE. Bazer FW. Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. Journal of Reproduction & Fertility. 75(1):69-78, 1985

Flood PF. The development of the conceptus and its relationship to the uterus. In Reproduction in Domestic Animals, (4th edition), ed. by P.T. Cupps. Academic Press, pp. 315-360, 1991.

Fortunato SJ. Menon R. Lombardi SJ. Expression of a progelatinase activator (MT1-MMP) in human fetal membranes. American Journal of Reproductive Immunology. 39(5):316-22, 1998

Gadsby JE. Heap RB. Burton RD. Oestrogen production by blastocyst and early embryonic tissue of various species. Journal of Reproduction & Fertility. 60(2):409-17, 1980

Geisert RD. Brookbank JW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biology of Reproduction. 27(4):941-55, 1982a

Geisert RD. Renegar RH. Thatcher WW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biology of Reproduction. 27(4):925-39, 1982b

Geisert RD. Yelich JV. Regulation of conceptus development and attachmen in pigs. Journal of Reproduction & Fertility - Supplement. 52:133-149, 1997.

Giambernardi TA. Grant GM. Taylor GP. Hay RJ. Maher VM. McCormick JJ. Klebe RJ. Overview of matrix metalloproteinase expression in cultured human cells. Matrix Biology. 16(8):483-96, 1998

Godkin JD. Bazer FW. Lewis GS. Geisert RD. Roberts RM. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biology of Reproduction. 27(4):977-87, 1982

Gomez DE. Alonso DF. Yoshiji H. Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. European Journal of Cell Biology. 74(2):111-22, 1997

Greene J. Wang M. Liu YE. Raymond LA. Rosen C. Shi YE. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. Journal of Biological Chemistry. 271(48):30375-80, 1996

Hampton AL. Butt AR. Riley SC. Salamonsen LA. Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. Biology of Reproduction. 53(2):302-11, 1995

Harvey MB. Leco KJ. Arcellana-Panlilio MY. Zhang X. Edwards DR. Schultz GA. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Development. 121(4):1005-14, 1995b

Heap RB. Flint AP. Hartmann PE. Gadsby JE. Staples LD. Ackland N. Hamon M. Oestrogen production in early pregnancy. Journal of Endocrinology. 89 Suppl:77P-94P, 1981

Hunter RHF. Chronological and cytological details of fertilization and early embryonic development in the domestic pig, *Sus scrofa*. Anatomical Record 178:169-86, 1974.

Hurskainen T. Hoyhtya M. Tuuttila A. Oikarinen A. Autio-Harmainen H. mRNA expressions of TIMP-1, -2, and –3 and 92-KD type IV collagenase in early human

placenta and decidual membrane as studied by in situ hybridization. Journal of Histochemistry & Cytochemistry. 44(12):1379-88, 1996

Itoh Y. Ito A. Iwata K. Tanzawa K. Mori Y. Nagase H. Plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface. Journal of Biological Chemistry. 273(38):24360-7, 1998

Jeffrey JJ. Interstitial collagenases. In Matrix Matalloproteinases, ed. by W. C. Parks and R. P. Mecham, Academic Press, San Diego, USA, 1998, pp. 15-42.

Kemp B. Soede NM. Consequences of variation in interval from insemination to ovulation on fertilization in pigs. Journal of Reproduction & Fertility - Supplement. 52:79-89, 1997

Keys JL. King GJ. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. American Journal of Anatomy. 188(3):221-38, 1990

Kinoh H. Sato H. Tsunezuka Y. Takino T. Kawashima A. Okada Y. Seiki M. MT-MMP, the cell surface activator of proMMP-2 (pro-gelatinase A), is expressed with its substrate in mouse tissue during embryogenesis. Journal of Cell Science. 109 (Pt 5):953-9, 1996

Lantz MS. Ciborowski P. Zymographic techniques for detection and characterization of microbial proteases. Methods in Enzymology. 235: 561-594, 1994.

Leco KJ. Apte SS. Taniguchi GT. Hawkes SP. Khokha R. Schultz GA. Edwards DR. Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues. FEBS Letters. 401(2-3):213-7, 1997

Leco KJ. Edwards DR. Schultz GA. Tissue inhibitor of metalloproteinases-3 is the major metalloproteinase inhibitor in the decidualizing murine uterus. Molecular Reproduction & Development. 45(4):458-65, 1996

Leco KJ. Khokha R. Pavloff N. Hawkes SP. Edwards DR. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. Journal of Biological Chemistry. 269(12):9352-60, 1994

Levenson AS. Kwaan HC. Svoboda KM. Weiss IM. Sakurai S. Jordan VC. Oestradiol regulation of the components of the plasminogen-plasmin system in MDA-MB-231 human breast cancer cells stably expressing the oestrogen receptor. British Journal of Cancer. 78(1):88-95, 1998

Librach CL. Werb Z. Fitzgerald ML. Chiu K. Corwin NM. Esteves RA. Grobelny D. Galardy R. Damsky CH. Fisher SJ. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. Journal of Cell Biology. 113(2):437-49, 1991

Mattson BA. Overstrom EW. Albertini DF. Transitions in trophectoderm cellular shape and cytoskeletal organization in the elongating pig blastocyst. Biology of Reproduction. 42(1):195-205, 1990

McMaster MT. Bass KE. Fisher SJ. Human trophoblast invasion. Autocrine control and paracrine modulation. Annals of the New York Academy of Sciences. 734:122-31, 1994

Menino AR Jr. Hogan A. Schultz GA. Novak S. Dixon W. Foxcroft GR. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. Developmental Genetics. 21(1):68-74, 1997

Morgan GL. Geisert RD. Zavy MT. Shawley RV. Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. Journal of Reproduction & Fertility. 80(1):125-31, 1987a

Morgan GL. Geisert RD. Zavy MT. Fazleabas AT. Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. Journal of Reproduction & Fertility. 80(1):133-41, 1987b

Morgan M. Kniss D. McDonnell S. Expression of metalloproteinases and their inhibitors in human trophoblast continuous cell lines. Experimental Cell Research. 242(1):18-26, 1998

Mullins DE. Bazer FW. Roberts RM. Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell. 20(3):865-72, 1980

Nagase H. Ogata Y. Suzuki K. Enghild JJ. Salvesen G. Substrate specificities and activation mechanisms of matrix metalloproteinases. Biochemical Society Transactions. 19(3):715-8, 1991

Papaioannou VE. Ebert KM. The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. Development. 102(4):793-803, 1988

Parsons SL. Watson SA. Brown PD. Collins HM. Steele RJ. Matrix metalloproteinases. British Journal of Surgery. 84(2):160-6, 1997

Patten BM. Embryology of the Pig (3rd edition). McGraw-Hill Book Company, New York, USA. 1948.

Perry JS. The mammalian fetal membranes. Journal of Reproduction & Fertility. 62(2):321-35, 1981

Pusateri AE. Rothschild MF. Warner CM. Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. Journal of Animal Science. 68(11):3727-35, 1990

Reponen P. Leivo I. Sahlberg C. Apte SS. Olsen BR. Thesleff I. Tryggvason K. 92-kDa type IV collagenase and TIMP-3, but not 72-kDa type IV collagenase or TIMP-1 or TIMP-2, are highly expressed during mouse embryo implantation. Developmental Dynamics. 202(4):388-96, 1995

Salamonsen LA. Role of proteases in implantation. Reviews of Reproduction. 4:11-22, 1999.

Salamonsen LA. Nagase H. Woolley DE. Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast-uterine interface. Journal of Reproduction and Fertility Supplement 49:29-37, 1995.

Samuel CA. Perry JS. The ultrastructure of pig trophoblast transplanted to an ectopic site in the uterine wall. Journal of Anatomy. 113(1):139-49, 1972a

Samuel CA. The development of pig trophoblast in ectopic sites. Journal of Reproduction & Fertility. 27(3):494-5, 1971

Samuel CA. Ultra-structural features of the ectopic pig trophoblast. Journal of Physiology. 226(2):22P, 1972b

Shimonovitz S. Hurwitz A. Dushnik M. Anteby E. Geva-Eldar T. Yagel S. Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. American Journal of Obstetrics & Gynecology. 171(3):832-8, 1994.

Soede NM. Kemp B. Expression of oestrus and timing of ovulation in pigs. Journal of Reproduction & Fertility - Supplement. 52:91-103, 1997

Soede NM. Helmond FA. Kemp B. Periovulatory profiles of oestradiol, LH and progesterone in relation to oestrus and embryo mortality in multiparous sows using transrectal ultrasonography to detect ovulation. Journal of Reproduction & Fertility. 101(3):633-41, 1994

Staskus PW, Masiarz FR, Pallanck LJ, Hawkes SP. The 21-kDa protein is a transformation-sensitive metalloproteinase inhibitor of chicken fibroblasts. J Biol Chem 1991; 266:449-54.

Stroband HW. Van der Lende T. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction & Fertility - Supplement. 40:261-77, 1990

Strongin AY. Collier I. Bannikov G. Marmer BL. Grant GA. Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. Journal of Biological Chemistry. 270(10):5331-8, 1995

Strongin AY. Marmer BL. Grant GA. Goldberg GI. Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. Journal of Biological Chemistry. 268(19):14033-9, 1993

Will H. Hinzmann B. cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. European Journal of Biochemistry. 231(3):602-8, 1995

Woessner JF Jr. Regulation of matrilysin in the rat uterus. Biochemistry & Cell Biology. 74(6):777-84, 1996

Woessner JF Jr. The family of matrix metalloproteinases. Annals of the New York Academy of Sciences. 732:11-21, 1994

Wolf K. Sandner P. Kurtz A. Moll W. Messenger ribonucleic acid levels of collagenase (MMP-13) and matrilysin (MMP-7) in virgin, pregnant, and postpartum uterus and cervix of rat. Endocrinology. 137(12):5429-34, 1996

Yelich JV. Pomp D. Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biology of Reproduction 57:1256-65, 1997

Zavy MT. Bazer FW. Thatcher WW. Wilcox CJ. A study of prostaglandin F2 alpha as the luteolysin in swine: V. Comparison of prostaglandin F, progestins, estrone and estradiol in uterine flushings from pregnant and nonpregnant gilts. Prostaglandins. 20(5):837-51, 1980

CHAPTER 4

EXPRESSION OF INTEGRIN TRANSCRIPTS IN BLASTOCYSTS DURING EARLY EMBRYOGENESIS, AND IN THE ENDOMETRIUM DURING EARLY GESTATION, IN THE PIG

INTRODUCTION

During pre-implantation embryonic development, a newly "hatched" porcine blastocyst consists of an inner cell mass (ICM) surrounded by a single layer of trophoblast, and has a spherical shape with a diameter of approximately 0.2 mm. It expands rapidly due to the inward transport of fluid by trophoblast cells. As the blastocyst expands, some of the ICM cells initiate an outgrowth along the inner surface of the trophoblast and subsequently form a complete layer of cells called the extraembryonic endoderm (Patten, 1948; Geisert et al., 1982a; Barends et al., 1989). Immediately after the endoderm has been established as a definite layer, the blastocyst undergoes a striking elongation from a spherical shape of about 8 to 10 mm in diameter to a filamentous shape of up to 100 cm in length (Geisert et al., 1982a). Along with the elongation process, the extraembryonic mesoderm initiates development from the primitive streak region and starts to migrate between the trophoblast and the extraembryonic endoderm (Patten, 1948). The trophoblast and the uterine epithelium become closely apposed to each other immediately after the blastocyst elongation is complete (Flood, 1991). This is followed by adhesion between these two cell layers around Day 14 of pregnancy, seen as the microvillous interdigitation between them by Day 16 of pregnancy (Day 0 designated as onset of estrus) (Dantzer, 1985; Keys and King, 1990). Implantation of the blastocyst is an essential step during mammalian pregnancy, leading to the formation of a placenta that supports embryonic/fetal development to term. Embryo implantation during pregnancy in

the pig is a non-invasive process resulting in simple attachment between the trophoblast and the uterine epithelium. This is in contrast to the invasive implantation seen in the mouse, where the trophoblast breaches the basement membrane of the uterine epithelium and penetrates into the underlying stromal tissue. It has been reported that one of the most important "windows" of embryonic mortality occurs from Day 13 to 20 of pregnancy in the pig, which is coincident with this embryo attachment process (Bazer, 1994). Therefore, elucidation of the mechanism(s) regulating the endodermal and mesodermal migration in blastocysts and the trophoblast-uterine epithelium attachment is essential for understanding aberrant processes of embryogenesis and/or implantation that may contribute to embryonic loss in this economically important domestic animal.

Early embryogenesis and attachment/implantation in the pig involves extensive cell-cell and cell-extracellular matrix interactions. These cellular interactions are mediated by several classes of adhesion molecules, one of the most important of which is the integrin family of cell surface adhesion receptors (Albelda and Buck, 1990; Hynes, 1992; Gille and Swerlick, 1996). Integrins function as transmembrane heterodimeric proteins consisting of α and β subunits linking cytoplasmic components of the cytoskeleton with the extracellular matrix (ECM) (Juliano, 1996; Yurochko, 1997). At least 24 integrin heterodimers can be formed from the 16 α subunits and 9 β subunits known, and nine of these have been detected in human uterine epithelium (Aplin, 1997). Integrins are modulated in the uterus during the reproductive cycle and early pregnancy in humans and rats (Nishida, Murakami and Otori, 1991; Lessey et al., 1992; Tabibzadeh, 1992; Lessey, 1997). Human and mouse embryos express specific integrins on their surface during pre-implantation development (Sutherland, Calarco and Damsky, 1993;

Campbell et al., 1995). The critical roles of integrins in differentiation, migration and invasion of trophoblast in both human and mouse have been reviewed by Damsky, Sutherland and Fisher (1993). Recently, Bowen and Hunt (1999) showed that integrins (α4, αv, β1 and β3) were also expressed within the developing murine placenta. As a first step in understanding the function of integrins during pregnancy in the pig, it was necessary to examine the expression and distribution of integrins in both the developing conceptus and in the female reproductive tract during pregnancy. Using indirect immunocytochemical techniques, the expression of selected integrin protein subunits was previously examined in porcine uterine epithelium and trophoblast *in vivo* and *in vitro* by Bowen, Bazer and Burghardt (1996 & 1997). Expression of integrin β1 subunit transcripts was previously examined in the early developing porcine conceptus by Yelich, Pomp and Geisert (1997a). Due to limitations on the amount of conceptus tissue during early embryogenesis, examination of the expression of gene transcripts in the whole embryo at this developmental stage has relied on the use of sensitive detection methods such as reverse-transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization (Rappolee et al., 1989; Yelich, Pomp, D. & Geisert 1997a & 1997b). In the present study, the RT-PCR approach was used to characterize the expression patterns of transcripts for eight integrin subunits in blastocysts during early embryogenesis, in Day-28 placental tissue, in cultured trophoblast cells (Jag-1), and in the endometrium during early gestation in the pig. Our results support an important role for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, and with two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of estrus (standing heat) with a vasectomized boar. At 16 and 24 hours after onset of estrus, gilts were artificially inseminated using fresh-pooled crossbred semen. All animals were handled in accordance with the Canadian Council on Animal Care (1993). Pregnant gilts were slaughtered as indicated below, and the reproductive tracts were recovered within 10 minutes of death.

B. Collection of Blastocysts, Endometrial and Placental Tissues

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed briefly in physiological saline to remove uterine debris. Spherical blastocysts were classified according to their sizes into three groups: 1-2 mm (n=37), 5-6 mm (n=19) and 8-10mm (n=8) in diameter, which were collected from gilts on Days 9 (n=3), 10 (n=2) and 10.5 (n=1) of pregnancy, respectively (Table 4.1). Blastocysts representing elongated stages were found to clump together, and it was difficult to separate individual blastocysts from each other after they were flushed from the reproductive tract. Therefore, elongated blastocysts were pooled for individual gilts obtained on Days 11, 12, 14 and 16 of pregnancy (Table 4.1). The number of elongated blastocysts obtained from each pig was estimated by counting the free bulbous ends of aggregated blastocysts.

Sections of endometrial tissue were harvested from the mid-section of each uterine horn of gilts slaughtered on Days 9, 10, 10.5, 11, 12, 14, 16 and 28 of pregnancy

(Table 4.1). Additionally, endometrial samples of Days 3, 5.5, 7, 7.5, 11.75, 12.75, 13.5, 13.75, 15.5, 15.75 of pregnancy were collected previously in our laboratory. Sections of placental tissue were obtained from the allantochorionic membrane distal to the embryonic region from Day 28 conceptuses. All tissue samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. Cell Culture and Neutrophil Collection

A porcine trophoblast cell line, Jag-1, was included for comparison of gene expression, and Jag-1 cells were cultured and harvested as described previously by Ramsoondar et al. (1993).

Porcine blood was collected at slaughter in tubes containing an anticoagulant (heparin) and centrifuged at 200g for 10 min. The plasma was collected and diluted 1:2 with PBS buffer containing 2% (w/v) BSA. Diluted plasma (3-4 ml) was carefully layered onto a Histopaque (Sigma) (HP) step gradient with 3 ml HP-1077 over 3 ml HP-1119 in a 15-ml conical centrifuge tube (Boyum, 1968). Two distinct opaque layers could be observed following centrifugation at 700g for 30 min at room temperature. The lower layer containing neutrophils was recovered by careful aspiration and diluted with the buffer in a fresh tube. The mixture was centrifuged at 200g for 10 min, and the pellet was resuspended and washed twice with the buffer to remove excess HP reagent.

D. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues, Jag-1 cells or neutrophils, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen

prior to the addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

E. Primers and RT-PCR

PCR primers for integrin subunits used in the present study were designed according to conserved regions between human and mouse cDNA sequences, and primers for G3PDH were synthesized as described by Yelich, Pomp and Geisert (1997). PCR primer sequences and their expected product sizes are listed in Table 4.2.

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 μg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 μl, containing 5 μM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% (v/v) Tween 20. Aliquots from the same RT reaction were used to amplify each of the target genes including G3PDH (Table 4.2) for each RNA sample. A master reaction mixture was prepared for PCR amplification of all target genes in that sample. Therefore, PCR amplification of all target genes for each sample was carried out under the same conditions, except that 0.4 μl of cDNA template were used for G3PDH while 5 μl of cDNA template were used for all integrin subunits. PCR was performed with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles (denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 min), and a final extension step at 72 °C for 7 min. All PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 Thermocycler in a reaction volume of 50 μl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 μM of each specific primer, 20 mM Tris-HCl pH 8.4,

50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. The identities of amplified products were confirmed by sequencing and/or diagnostic restriction digestion analysis. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes (N lanes in Fig. 4.1 and Fig. 4.2).

RESULTS

A. Expression of Integrin Transcripts in Blastocysts

Transcripts of integrin subunits (α1, α2, α3, α4, α5, αv, β1 and β3) were examined in spherical and elongated blastocysts collected between Day 9 and 16 of pregnancy. In blastocysts at all stages examined, expression of G3PDH transcripts was both evident and relatively constant (Fig. 4.1A). Expression of α2, α3, αv, β1 and β3 transcripts exhibited similar overall patterns in blastocysts during the period examined (Fig. 4.1C-G). Expression was high in spherical blastocysts (1-2 mm in diameter; Lane S1), was dramatically down-regulated in spherical blastocysts (5-6 mm and 8-10 mm; Lanes S2 and S3), and progressively increased as blastocysts elongated between Days 11 and 16 (Lanes F1 to F4). It is interesting to note that transcripts of all integrin subunits examined were not detected in the 8-10 mm spherical blastocysts, even though levels of G3PDH transcripts were comparable to blastocysts at other developmental stages (Fig. 4.1A-G). In order to increase the sensitivity of PCR detection, a higher number (45) of PCR cycles was used for amplification of the integrin subunit transcripts. When this was done, low levels of αv, β1 and β3 transcripts could be detected in the 8-10 mm spherical blastocysts, whereas transcripts of other integrin subunits were still not observed (data

not shown). The data in Figure 4.1 also suggest variation in the pattern of up-regulation of this group of integrin subunits between Days 11 and 16 of pregnancy; expression of α3, αv, β1 and β3 transcripts appeared to be progressively up-regulated (Fig. 4.1D-G), in contrast to variable expression of α2 transcripts during this period (Fig. 4.1C). The pattern of expression of α1 transcripts was unique in blastocysts, being undetectable in three spherical stages examined and only observed in elongated blastocysts collected on Days 14 and 16 (Fig. 4.1B). In contrast, expression of α4 and α5 transcripts was not observed in either spherical or elongated blastocysts examined even when a higher number (45) of PCR cycles were used for amplification (data not shown).

B. Expression of Integrin Transcripts in the Placental Tissue and Jag-1 Cells

In the placental tissue (Lane P in Fig. 4.1) collected on Day 28 of pregnancy, expression of α1, α2, α3, αv, β1 and β3 transcripts was observed. The α1, α3, αv and β3 subunits were moderately expressed (Fig. 4.1B, D, E & G), while expression of α2 and β1 transcripts was observed at relatively low levels (Fig. 4.1C & F). In contrast, Jag-1 cells (Lane T in Fig. 4.1) expressed high levels of α1, α2, α3, αv and β3 transcripts (Fig. 4.1B-D, E & G) and low levels of β1 transcripts (Fig. 4.1F). Transcripts for the α5 subunit were detected at low levels in the Jag-1 cells and in the placental tissues (data not shown). Transcripts of α4 subunits were undetectable in either the placental tissues or the Jag-1 cells, although they could be detected in porcine neutrophils (data not shown).

C. Expression of Integrin Transcripts in the Endometrium

G3PDH transcripts could be detected in all endometrial tissues examined (Fig. 4.2A). Four distinct expression patterns of integrin transcripts were observed in porcine

endometrial tissues obtained between Day 3 and 28 of pregnancy. Firstly, relatively constant and high levels of expression for αv, β1 and β3 transcripts were observed in all endometrial tissues obtained (Fig. 4.2F, G & H). Secondly, high-level and developmentally regulated expression was observed for α1 and α2 transcripts. Transcripts for α1 were low in endometrial tissues obtained between Days 3 and 9 of pregnancy (Fig. 4.2B), and remained relatively high in endometrial tissues obtained between Days 10 and 28 of pregnancy except Day 14 of pregnancy (Fig. 4.2B). Expression of α2 transcripts exhibited a variable pattern in endometrial tissues during the period examined (Fig. 4.2C). Thirdly, low-level modulated expression was observed for α3 and α5 transcripts. Expression levels of α3 transcripts were low in endometrial tissues collected between Days 3 and 11.75 of pregnancy (Fig. 4.2D), transiently increased in endometrial tissues obtained around Day 12 of pregnancy (Fig. 4.2D), and were not detected between Days 12.75 and 28 of pregnancy (Fig. 4.2D). Expression of α5 transcripts was observed at very low levels in endometrial tissues obtained between Days 3 and 7.5 of pregnancy (Fig. 4.2F), and was not detected in endometrial tissues collected between Days 11.75 and 28 of pregnancy (Fig. 4.2F). Fourthly, expression of α4 transcripts was not detectable in any endometrial tissues examined (data not shown).

DISCUSSION

During early mammalian embryogenesis, the segregation and differentiation of extraembryonic cell lineages is extremely important for the embryo to sustain its growth and development. Integrins have been shown to play a critical role in early embryogenesis, implantation and placentation in the mouse (Damsky, Sutherland and

Fisher, 1993). Mouse embryos lacking the β1 subunit gene have been shown to develop normally to the blastocyst stage but fail to implant (Fasser and Meyer, 1995; Stephens et al., 1995). It is generally believed that visceral and parietal (or extraembryonic) endoderm cells both arise from primitive endodermal cells (Gardner, 1983; Rosenstraus, Spadoro and Nilsson, 1983). In porcine blastocysts, primitive endodermal cells first appear on the inner face of the ICM on Day 7 of pregnancy, and some of these cells subsequently migrate along the inner face of the trophoblast to give rise to the extraembryonic endoderm (Stroband, Taverne and v an der Bogaard, 1984). On Day 8, extraembryonic endodermal cells have been observed on the inner face of the trophoblast in expanding blastocysts (Richoux et al., 1989). It is probable that the migratory activity of extraembryonic endodermal cells is very high in the expanding spherical blastocysts of 1-2 mm in diameter collected on Day 9 in the present study. Extracellular deposition of fibronectins (FN) was shown to be abundant on the whole inner face of the trophoblast during, and even before, the extraembryonic endodermal migration (Richoux et al., 1989). It is known that FN is a ligand for α3β1, αvβ1 and αvβ3 integrins (Aplin, 1997). Therefore, it is not surprising that high levels of transcripts have been observed for α2, α3, αv, β1 and β3 in these spherical blastocysts. These subunits could potentially form α2β1, α3β1, αvβ1 and αvβ3 functional heterodimers. These data suggest that extraembryonic endodermal migration in porcine blastocysts might be mediated through interactions between integrins α3β1, αvβ1 and αvβ3 possibly expressed on the cell surface of endodermal cells and FN ligands present on the whole inner face of the trophoblast. In contrast, it is interesting to note in the present study that expression of α2, α3, αv, β1 and β3 transcripts was dramatically down regulated in spherical blastocysts

(5-6 mm and 8-10 mm in diameter). These data suggest that cellular migratory activities might be low at these two developmental stages. Indeed, previous morphological observations have shown that the extraembryonic endoderm layer is almost completely established in spherical blastocysts at these developmental stages while the development of extraembryonic mesoderm has still not been initiated (Geisert et al., 1982a; Richoux et al., 1989; Gupta, Bazer and Jaeger, 1996). Therefore, there might be no need for blastocysts at these developmental stages to express high levels of integrin transcripts.

Upon reaching a size of about 10 mm in diameter around Day 11.5 of pregnancy, porcine blastocysts undergo a rapid transition from spherical to elongated forms, which is coincident with the differentiation and initial migration of extraembryonic mesoderm (Geisert et al., 1982a; Gupta, Bazer and Jaeger, 1996). During this initial elongation process, physiological activities of blastocysts are increased as seen by the onset of embryonic estrogen and protein synthesis (Gadby, Heap and Burton, 1980; Godkin et al., 1982; Fischer, Bazer and Fields, 1985). These observations are extended by our findings that expression of $\alpha 2$, $\alpha 3$, αv, $\beta 1$ and $\beta 3$ transcripts increase dramatically in elongated blastocysts obtained between Days 11 and 16. Our data on the expression of αv, $\beta 1$ and $\beta 3$ transcripts in blastocysts are consistent with the previous report of the expression of αv, $\beta 1$ and $\beta 3$ proteins in the trophoblast and implantation sites between Days 11 to 15 of pregnancy (Bowen, Bazer and Burghardt, 1996). Our temporal expression profiles of $\beta 1$ transcripts in blastocysts are also consistent with a previous study, in which expression of $\beta 1$ transcripts tended to increase from spherical to filamentous blastocysts obtained from Day 10 to 12 of pregnancy (Yelich, Pomp and Geisert, 1997a). High levels of $\alpha 3$ transcripts detected in the present study might be

mainly expressed in endodermal cells, since α3 proteins were only localized in extraembryonic endodermal cells but were not seen in trophoblast cells of blastocysts obtained between Day 11 to 15 of pregnancy (Bowen, Bazer and Burghardt, 1996).

Two essential processes occur in porcine blastocysts between Day 11 to 16 of pregnancy: the differentiation and migration of extraembryonic mesodermal cells and the initial attachment between the trophoblast and the uterine epithelium (Geisert et al., 1982a; Pusateri et al., 1990; Gupta, Bazer and Jaeger, 1996; Wilson and Ford, 1997). Cell migration requires a reduction in cell contacts, and is generally accompanied by a loss of expression or function of cell adhesion molecules. Changes in the level of α2β1 expression is correlated with the initiation of tumor cell migration in many breast cancers (Zutter and Santoro, 1998; Alford, Pitha-Rowe and Taylor-Papadimitriou, 1998). High levels of the α2β1 integrin ligand, collagen type IV, was shown to be present at the basal laminae of both the trophoblast and the uterine epithelium during early pregnancy in the pig (Bowen, Bazer and Burghardt, 1996). In the present study, expression of α2 transcripts has been shown to be regulated in blastocysts obtained between Days 9 and 16 of pregnancy, which is temporally associated with extraembryonic endodermal and mesodermal cell migration in the pig. In addition, the fluctuation of α2 transcript levels is also observed in porcine endometrial tissues obtained during early gestation in the present study. Taken together, these results suggest that α2β1 integrin might play an important role in the differentiation, proliferation and migration of both extraembryonic lineages and the uterine epithelium during early gestation in the pig.

Attachment between the trophoblast and the uterine epithelium starts on Day 13 to 14 of pregnancy in the pig (Dantzer, 1985). The present study demonstrates that

expression of α_1 transcripts is not detected in porcine blastocysts until Day 14 of pregnancy. Furthermore, expression levels of α_1 transcripts were very high in Day 28 placental tissue and in trophoblast cells (Jag-1) derived from Day 14 blastocysts. It is also interesting to note in the current study that expression of α_1 transcripts was higher in endometrial tissues obtained at the time of embryo-uterine contact compared to other developmental stages. These results extend previous observations on the contact-inductive nature of α_1 expression during embryo implantation in humans and mice, where α_1 expression on the trophoblast is a response to trophoblast contact with ECM in the uterus (Damsky, Fitzgerald and Fisher, 1992; Sutherland, Calarco and Damsky 1993). Integrin $\alpha_1\beta_1$ is a known receptor for collagen and laminin (Forsberg et al., 1990; Lessey, 1997). However, the α_1 proteins, as well as type IV collagen and laminin were demonstrated to localize only at the basal laminae of both the uterine epithelium and the trophoblast at implantation sites in the pig (Bowen, Bazer and Burghardt, 1996; Burghardt et al., 1997). As such, $\alpha_1\beta_1$ integrin would not be appropriately positioned to participate in adhesive interactions between the apical surfaces of the trophoblast and the uterine epithelium (Burghardt et al., 1997). However, the integrin $\alpha_1\beta_1$ might play a role in stabilizing the adhesion interactions between the trophoblast and its underlying basement membrane (BM) and between the uterine epithelium and its BM, respectively. Intact and firm trophoblast and uterine epithelial layers are essential for supporting the subsequent extraembryonic mesodermal migration and the extensive morphological remodeling in blastocysts and the endometrium, during the attachment/ implantation process in the pig, such as the formation of trophoblastic caps and endometrial protrusions (Dantzer, 1985; Keys and King, 1990). Furthermore, expression of α_1

transcripts was not detected in small spherical blastocysts (1-2 mm in diameter) in the present study, which suggests that integrin $\alpha_1\beta_1$ is not likely to be involved in the extraembryonic endodermal cell migration in the pig. Integrin $\alpha_3\beta_1$ is a cell surface receptor for collagen, laminin and FN (Lessey, 1997), all of which have been shown to be present in the ECM underlying the trophoblast (Richoux et al., 1989; Bowen, Bazer and Burghardt, 1996; Burghardt et al., 1997). Unlike the α_1 subunit, expression of α_3 transcripts was detected in the present study in small spherical blastocyst (1-2 mm in diameter). In addition, expression of α_3 transcripts increased in elongated blastocysts obtained between Days 11 and 16, which correlates with the initiation and migration of extraembryonic mesoderm in the pig. However, expression of α_3 proteins was restricted to the basal lamina of the uterine epithelium, and was detected in the endoderm but not in the trophoblast of blastocysts during early pregnancy in the pig (Bowen, Bazer and Burghardt, 1996). Taken together, we hypothesize that integrin $\alpha_3\beta_1$ might be directly involved in extraembryonic endodermal and mesodermal cell migration in the pig.

In the present study, similar expression patterns of β1 and β3 transcripts were observed both in the placental tissues at Day 28 and in Jag-1 cells, with β3 levels being higher in both cases. In contrast, Bowen and Hunt (1999) showed that expression of β1 at the mRNA and protein levels was higher than that of β3 in mouse placenta, which is known to be invasive during embryo implantation. In addition, expression of the β1 subunit was shown to be up regulated during trophoblast invasion in human uterus (Aplin, 1997). Furthermore, β1-knockout mouse embryos develop normally to the blastocyst stage but fail to sustain implantation (Fassler and Meyer, 1995; Stephens et al., 1995). Collectively, those results are consistent with a role for β1 integrins in the invasive

type of implantation seen in the human and mouse. In contrast, low-level expression of the β1 subunit in the porcine placenta might contribute to the non-invasive type of embryo implantation seen in the pig. High-level expression of β3 transcripts observed in the present study might be required for the adhesion between trophoblast cells in porcine blastocysts, which is supported by a previous observation by Bowen, Bazer and Burghardt (1996) that β3 proteins were restricted to the intercellular borders between trophoblast cells. Therefore, an intact layer of trophoblast cells could be maintained to ensure the integrity of the non-invasive epitheliochorial placenta in the pig.

The high-level expression of α_v, β_1 and β_3 transcripts observed in endometrial tissues during early gestation in the present study is consistent with the previous observation of α_v, $\beta_1$and β_3 protein expression in the uterine epithelium by Bowen, Bazer and Burghardt (1996). Integrin $\alpha_v\beta_3$ is a receptor for both FN and vitronectin (VN) (Hynes, 1992; Aplin, 1997). Bowen, Bazer and Burghardt (1996) demonstrated that both FN and VN were present on apical surfaces of the trophoblast and the uterine epithelium during early pregnancy in the pig. In addition, they found that both α_v and β_3 subunits were localized on the apical surface of the uterine epithelium during early pregnancy and at implantation sites, but were restricted to intercellular borders between trophoblast cells between Days 11 to 15 of pregnancy in the pig. These results suggest that interactions between the integrin $\alpha_v\beta_3$ on the uterine epithelium and its ligands (FN and VN) on the apical surface of the trophoblast might play a major role during embryo implantation in the pig. Since conceptus/fetal development in the pig relies on a non-invasive epitheliochorial placenta, firm adhesion between the trophoblast and the uterine

epithelium is essential to establish and sustain an efficient interface for nutrient and waste exchange with the maternal system.

Expression of integrin subunits α_4 and α_5 at the fetal/maternal interface appears to be a species-specific phenomenon. Expression of both α_4 and α_5 subunits was absent in human pre-implantation embryos (Campbell et al., 1995). Expression of α_5 proteins was only localized to the uterine stroma and not the uterine epithelium by Lessey (1994), although a number of discrepancies exist in the literature concerning the endometrial expression of α_5 subunit (Lessey et al., 1992; Tabibzadeh, 1992; Klentzeris et al., 1993). In contrast, expression of α_5 but not α_4 subunits was observed in mouse pre-implantation embryos (Sutherland, Calarco and Damsky, 1993). Furthermore, implantation defects were not observed in mouse embryos lacking α_4 and α_5 (Yang, Rayburn and Hynes, 1993 & 1995), suggesting that they are not involved in the implantation processes. In the present study, α_4 and α_5 transcripts were not detected in blastocysts obtained between Days 9 and 16 using the RT-PCR approach, although expression of α_4 and α_5 could be detected in porcine neutrophils and in Jag-1 cells, respectively, which were used as positive controls. Moderate and intense staining of α_4 and α_5 was previously reported in porcine uterine epithelium and trophoblast between Days 11 to15 of pregnancy and also at attachment points between the uterine epithelium and the trophectoderm (Bowen, Bazer and Burghardt, 1996). Considering the sensitivity of the RT-PCR approach, the transcript levels of α_4 and α_5 subunits detected in the present study are unlikely to produce the high levels of proteins in the uterine epithelium and in the trophoblast observed in the previous study by Bowen, Bazer and Burghardt (1996). Therefore, further clarification of the expression of α_4 and α_5 subunits during early pregnancy in the pig is

needed. In addition, it is obvious that there is a limitation on the number of animals used in the experiment examining integrin expression in blastocysts, although the number of blastocysts is resonably sufficient for representing individual stages of embryo development examined.

In summary, in the present study, the expression of eight integrin subunit (α1, α2, α3, α4, α5, αv, β1 and β3) transcripts was determined in blastocysts during early embryogenesis and in the endometrium during early gestation in the pig. Our data suggest that integrins might play important regulatory roles in early embryogenesis, implantation/attachment and placentation in the pig.

Table 4.1 Blastocsyt and placental tissue samples used in this experiment.

Sample	Day of pregnancy	Number and developmental stage of blastocysts and placental tissue	Number of pigs slaughtered
S1	9	37; Spherical (1-2 mm in diameter)	3
S2	10	19; Spherical (5-6 mm in diameter)	2
S3	10.5	8; Spherical (8-10 mm in diameter)	1
F1	11	8; Elongated blastocysts	1
F2	12	9; Elongated blastocysts	1
F3	14	12; Elongated blastocysts	1
F4	16	6; Elongated blastocysts	1
P	28	Placental tissue	1

Table 4.2 PCR primer sequences and predicted product sizes for integrin subunits#.

Target gene	Primer sequence	Size (bp)	Source*
G3PDH	5' ACCACAGTCCATGCCATCAC 3'	452	Yelich et al., 1997
	5' TCCACCACCCTGTTGCTGTA 3'		
α1	5' CTTCAATGTCTCTGATGAATTGGC 3'	975	AB000470; X68742;
	5' GAAGAGGGCAGCACCACCAAGGC 3'		X52140
α2	5' GGAGGAGACAACTTTCAGATGG 3'	1,206	M28249; Z29987
	5' GGGCAGGGCTAGTGCCAGGG 3'		
α3	5' GACTGGTGCTGTGTACCTGTG 3'	790	M59911; D13867
	5' CCCGCCTGGTTCATGAAGACATA 3'		
α4	5' GCCCTAATGGAGAACCTTGTGG 3'	1,264	L12002; X53176
	5' CTCTGCCTTTCTGTTCACATCC 3'		
α5	5' CTCAGGACTCGTCAGACACCC 3'	881	M74954; M74116;
	5' CCATAGCTGCCTTCTGCCTTGG 3'		X79003
αv	5' GCAGATGTGTTTATTGGAGCACC 3'	1,065	M14648; J02826;
	5' GAGCTCAGCTTCGTAGGCACC 3'		M18365; U14135
β1	5' GACCTCTACTACCTTATGGACC 3'	1,900	X07979; U12309
	5' GGATTTTCACCCGTGTCCCA 3'		Y00769
β3	5' CTACCCTGTGGACATCTACTAC 3'	792	U95204; X72378
	5' GGCATTGAAGGACAGCGACAGCTC 3'		M35999; M20311

#All primer sequences were listed from 5' end to 3' end. * Primers were designed according to conserved regions of human and mouse cDNA sequences obtained from GenBank database unless indicated. GenBank access numbers or references were listed.

Figure 4.1 RT-PCR amplification of G3PDH (A) and integrin subunits α_1 (B), α_2 (C), α_3 (D), α_v (E), β_1 (F) and β_3 (G) in blastocysts, placental tissues and Jag-1 cells. Total RNA was extracted from blastocysts from gilts representing Days 9 (S1), 10 (S2), 10.5 (S3), 11 (F1), 12 (F2), 14 (F3), and 16 (F4) of pregnancy, respectively, placental tissues of Day 28 (P) and trophoblast cells (Jag-1) (T). M, 100 bp DNA ladders. N, negative control. Names and predicted sizes of target genes are indicated to the right and left sides of the figures, respectively. PCR products were run in 1.2% (w/v) agarose gel, and gels were stained with ethidium bromide.

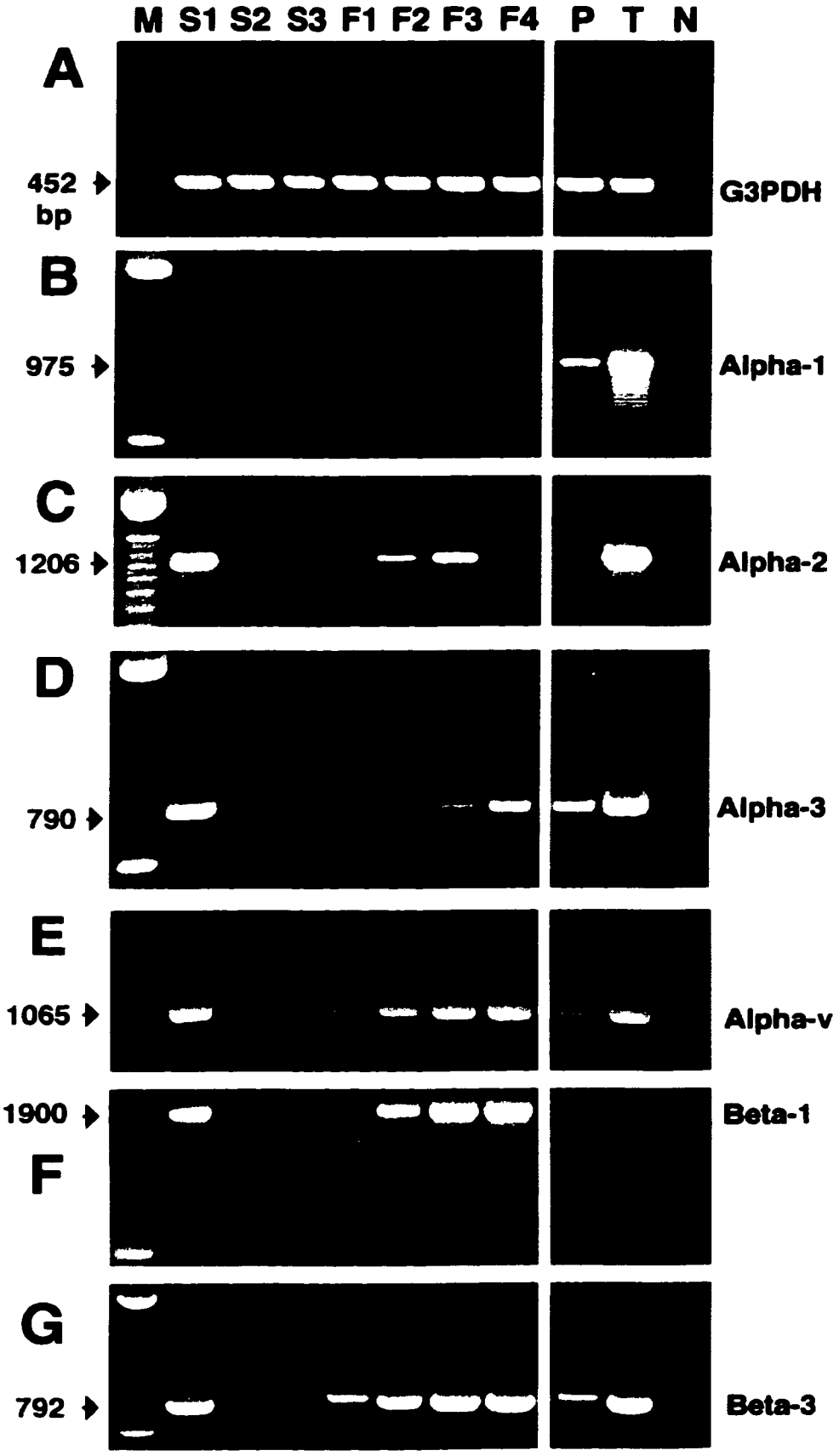
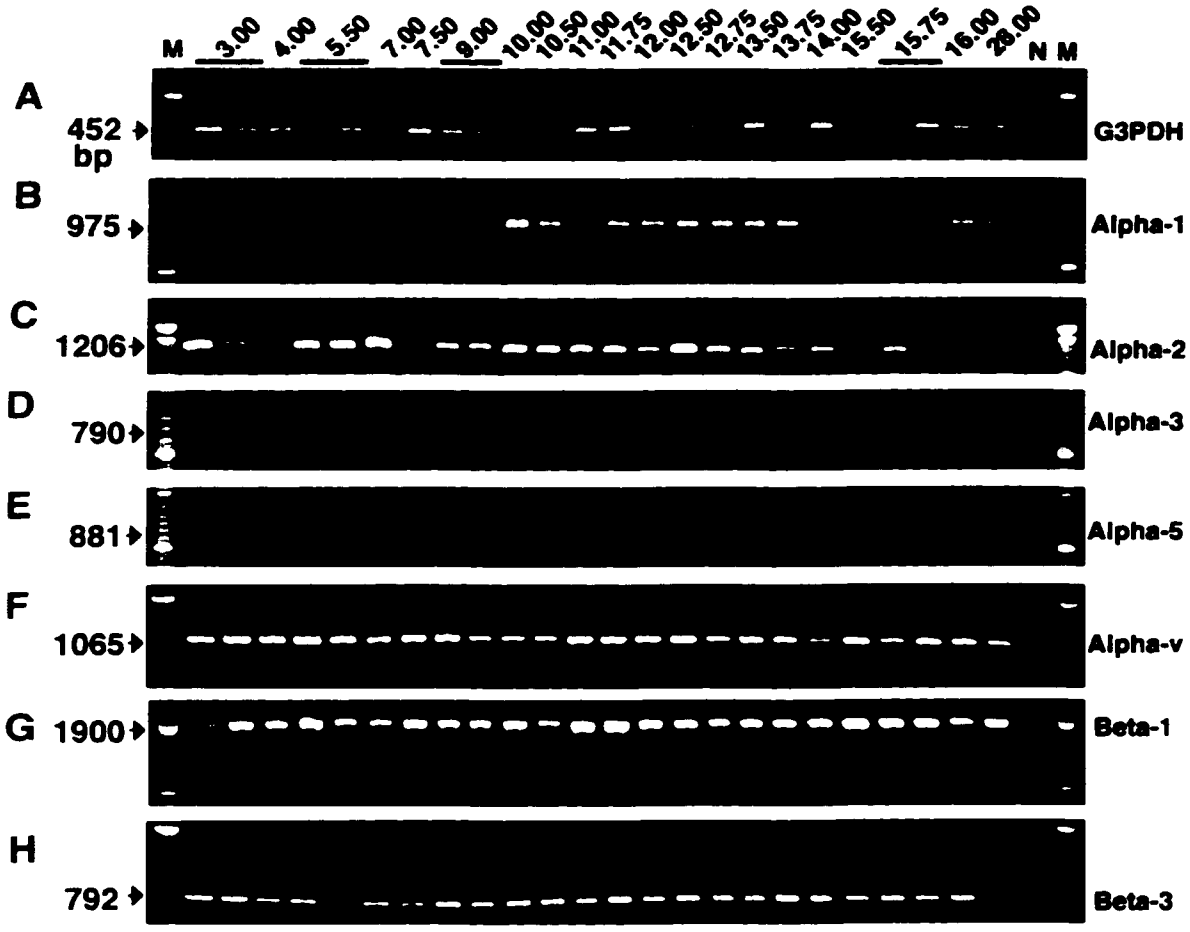

M S1 S2 S3 F1 F2 F3 F4 P T N
A
452 bp
G3PDH
B
975
Alpha-1
C
1206
Alpha-2
D
790
Alpha-3
E
1065
Alpha-v
1900
Beta-1
F
G
792
Beta-3

Figure 4.2 RT-PCR amplification of G3PDH (A), α_1 (B), α_2 (C), α_3 (D), α_5 (E), α_v (F), β_1 (G) and β_3 (H) in the endometrium during early gestation. Total RNA was extracted from endometrial tissues obtained from individual gilts on Days 3, 3, 4, 5.5, 5.5, 7, 7.5, 9, 9, 10, 10.5, 11, 11.75, 12, 12 .5, 12.75, 13.5, 13.75, 14, 15.5, 15.75, 15.75, 16 and 28 of pregnancy, respectively. M, 100 bp DNA ladders. N, negative control. Names and predicted sizes of target genes are indicated to the right and left sides of the figures, respectively. PCR products were run in 1.2% (w/v) agarose gel, and gels were stained with ethidium bromide.


M
3.00
4.00
5.50
7.00
7.50
8.00
10.00
10.50
11.00
11.75
12.00
12.50
12.75
13.50
13.75
14.00
15.50
15.75
16.00
28.00
N
M
A
452 bp
G3PDH
B
975
Alpha-1
C
1206
Alpha-2
D
790
Alpha-3
E
881
Alpha-5
F
1065
Alpha-v
G
1900
Beta-1
H
792
Beta-3

REFERENCES

Albelda SM. Buck CA. Integrins and other cell adhesion molecules. FASEB Journal. 4(11):2868-80, 1990

Alford D. Pitha-Rowe P. Taylor-Papadimitriou J. Adhesion molecules in breast cancer: role of alpha 2 beta 1 integrin. Biochemical Society Symposia. 63:245-59, 1998

Aplin JD. Adhesion molecules in implantation. Rev Reprod. 2(2):84-93, 1997

Barends PM. Stroband HW. Taverne N. Kronnie G. Leen MP. Blommers PC. Integrity of the preimplantation pig blastocyst during expansion and loss of polar trophectoderm (Rauber cells) and the morphology of the embryoblast as an indicator for developmental stage. Journal of Reproduction & Fertility. 87(2):715-26, 1989

Bazer FW. New frontiers and approaches in the study of embryonic mortality in domestic species. In Embryonic Mortality in Domestic Species, ed. by Michael T. Zavy and Rodney D. Geisert, CRC Press, Boca Raton, Florida, USA. Pg. 195-210, 1994

Bowen JA. Hunt JS. Expression of cell adhesion molecules in murine placentas and a placental cell line. Biology of Reproduction. 60:428-434, 1999

Bowen JA. Bazer FW. Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biology of Reproduction. 56(2):409-15, 1997

Bowen JA. Bazer FW. Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biology of Reproduction. 55(5):1098-106, 1996

Boyum A. Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest: Suppl 97:7, 1968.

Brooks PC. Stromblad S. Sanders LC. von Schalscha TL. Aimes RT. Stetler-Stevenson WG. Quigley JP. Cheresh DA. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell.85(5):683-93, 1996

Burghardt RC. Bowen JA. Newton GR. Bazer FW. Extracellular matrix and the implantation cascade in pigs. Journal of Reproduction & Fertility - Supplement. 52:151-64, 1997.

Campbell S. Swann HR. Seif MW. Kimber SJ. Aplin JD. Cell adhesion molecules on the oocyte and preimplantation human embryo. Human Reproduction. 10(6):1571-8, 1995

Damsky C. Sutherland A. Fisher S. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. FASEB Journal. 7(14):1320-9, 1993

Damsky CH. Fitzgerald ML. Fisher SJ. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. Journal of Clinical Investigation. 89(1):210-22, 1992

Dantzer V. Electron microscopy of the initial stages of placentation in the pig. Anatomy & Embryology. 172(3):281-93, 1985

Fassler R. Meyer M. Consequences of lack of beta 1 integrin gene expression in mice. Genes & Development. 9(15):1896-908, 1995

Fischer HE. Bazer FW. Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. Journal of Reproduction & Fertility. 75(1):69-78, 1985

Flood PF. The development of the conceptus and its relationship to the uterus. In Reproduction in Domestic Animals, (4th edition), ed. by P.T. Cupps. Academic Press, pp. 315-360, 1991.

Forsberg E. Paulsson M. Timpl R. Johansson S. Characterization of a laminin receptor on rat hepatocytes. Journal of Biological Chemistry. 265(11):6376-81, 1990

Gadsby JE. Heap RB. Burton RD. Oestrogen production by blastocyst and early embryonic tissue of various species. Journal of Reproduction & Fertility. 60(2):409-17, 1980

Gardner RL. Origin and differentiation of extraembryonic tissues in the mouse. International Review of Experimental Pathology.24:63-133, 1983

Geisert RD. Brookbank JW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biology of Reproduction. 27(4):941-55, 1982a

Geisert RD. Renegar RH. Thatcher WW. Roberts RM. Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biology of Reproduction. 27(4):925-39, 1982b

Gille J. Swerlick RA. Integrins: role in cell adhesion and communication. Annals of the New York Academy of Sciences. 797:93-106, 1996

Godkin JD. Bazer FW. Lewis GS. Geisert RD. Roberts RM. Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. Biology of Reproduction. 27(4):977-87, 1982

Gupta A. Bazer FW. Jaeger LA. Differential expression of beta transforming growth factors (TGF beta 1, TGF beta 2, and TGF beta 3) and their receptors (type I and type II) in peri-implantation porcine conceptuses. Biology of Reproduction. 55(4):796-802, 1996

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 69(1):11-25, 1992

Juliano R. Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. Bioessays. 18(11):911-7, 1996

Keys JL. King GJ. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. American Journal of Anatomy. 188(3):221-38, 1990

Klentzeris LD. Bulmer JN. Trejdosiewicz LK. Morrison L. Cooke ID. Beta-1 integrin cell adhesion molecules in the endometrium of fertile and infertile women. Human Reproduction. 8(8):1223-30, 1993

Lessey BA. Integrins and the endometrium: new markers of uterine receptivity. Annals of the New York Academy of Sciences. 828:111-22, 1997

Lessey BA. Castelbaum AJ. Buck CA. Lei Y. Yowell CW. Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertility & Sterility. 62(3):497-506, 1994

Lessey BA. Damjanovich L. Coutifaris C. Castelbaum A. Albelda SM. Buck CA. Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. Journal of Clinical Investigation. 90(1):188-95, 1992

Nishida T. Murakami J. Otori T. Expression of fibronectin receptor (integrin) in the uterus of rats in relation to the estrous cycle. Histochemistry. 96(4):279-83, 1991

Patten BM. Embryology of the Pig (3rd edition). McGraw-Hill Book Company, New York, USA. 1948.

Pusateri AE. Rothschild MF. Warner CM. Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. Journal of Animal Science. 68(11):3727-35, 1990

Rappolee DA. Wang A. Mark D. Werb Z. Novel method for studying mRNA phenotypes in single or small numbers of cells. Journal of Cellular Biochemistry. 39(1):1-11, 1989

Richoux V. Darribere T. Boucaut JC. Flechon JE. Thiery JP. Distribution of fibronectins and laminin in the early pig embryo. Anatomical Record. 223(1):72-81, 1989

Ramsoondar J. Christopherson RJ. Guilbert LJ. Wegmann TG. A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biology of Reproduction. 49(4):681-94, 1993

Rosenstraus MJ. Spadoro JP. Nilsson J. Cell position regulates endodermal differentiation in embryonal carcinoma cell aggregates. Developmental Biology. 98(1):110-6, 1983

Stephens LE. Sutherland AE. Klimanskaya IV. Andrieux A. Meneses J. Pedersen RA. Damsky CH. Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes & Development. 9(15):1883-95, 1995

Stroband HW. Taverne N. v.d. Bogaard M. The pig blastocyst: its ultrastructure and the uptake of protein macromolecules. Cell & Tissue Research. 235(2):347-56, 1984

Sutherland AE. Calarco PG. Damsky CH. Developmental regulation of integrin expression at the time of implantation in the mouse embryo. Development. 119(4):1175-86, 1993

Sutherland AE. Calarco PG. Damsky CH. Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth. Journal of Cell Biology. 106(4):1331-48, 1988

Tabibzadeh S. Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. Human Reproduction. 7(6):876-82, 1992

Wilson ME. Ford SP. Differences in trophectoderm mitotic rate and P450 17alpha-hydroxylase expression between late preimplantation Meishan and Yorkshire conceptuses. Biology of Reproduction. 56(2):380-5, 1997

Yelich JV. Pomp D. Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biology of Reproduction. 57(5):1256-65, 1997a

Yelich JV. Pomp D. Geisert RD. Detection of transcripts for retinoic acid receptors, retinol-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. Biology of Reproduction. 57(2):286-94, 1997b

Yurochko AD. Adhesion molecules as signal transduction molecules. In Adhesion molecules in Health and Disease, ed. by Paul LC, Issekutz TB. Marcel Dekker, Inc., New York, 1997.

Yang JT. Rayburn H. Hynes RO. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development. 121(2):549-60, 1995

Yang JT. Rayburn H. Hynes RO. Embryonic mesodermal defects in alpha 5 integrin-deficient mice. Development. 119(4):1093-105, 1993

Yelich JV. Pomp D. Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biology of Reproduction 57:1256-65, 1997

Yurochko AD. Adhesion molecules as signal transduction molecules. In Adhesion molecules in Health and Disease, ed. by Paul LC. Issekutz TB., Marcel Dekker, Inc., New York, 1997

Zutter MM. Santoro SA. The ups and downs of alpha 2 beta 1-integrin expression: contributions to epithelial cell differentiation and the malignant phenotype. Current Topics in Microbiology & Immunology. 231:167-85, 1998

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Considerable embryonic losses occur from Days 13 to 20 of pregnancy in the pig, which is coincident with mesodermal migration in blastocysts, blastocyst elongation and the trophoblast-uterine epithelium attachment (implantation). Little is known about the mechanisms regulating these processes in the pig, although great progress has been made in understanding molecular and cellular processes during embryo implantation and placental development in the mouse during the last decade (Schultz and Edwards, 1997). Therefore, there is a need to characterize the expression of important factors in the embryo and in the uterus during early gestation, especially during the peri-implantation period, in the pig. To date, a variety of soluble and membrane-associated factors that are relevant to the implantation process in other species have been shown to be present in blastocysts and the uterus during the peri-implantation period in the pig (Geisert and Yelich, 1997; Burghardt et al., 1997).

In the first experiment of the present study, we extended earlier findings of Ramsoondar et al. (1993) in which a porcine trophoblast cell line (Jag-1) was found to secrete growth factor(s) stimulating the proliferation of porcine macrophages. In the present study, serum-free conditioned media of Jag-1 exhibited a stimulatory effect on the proliferation of porcine macrophages in a dose-dependent fashion. However, a specific anti-CSF-1 hybridoma supernatant YY106 was able to abrogate almost completely this stimulatory activity. This result strongly suggests that CSF-1 is the major growth factor secreted by JAG-1 trophoblast cells, and is responsible for the observed bioactivity. Subsequently, the coding sequence of a porcine CSF-1 cDNA was

characterized, which showed significant homologies with human and mouse CSF-1 cDNA sequences. In addition, the CSF-1 gene was demonstrated to be present as a single copy in the pig genome, which extended earlier observations on the CSF-1 gene in human and mouse (Kawasaki et al., 1985; Ladner et al., 1987; Rajavashisth et al., 1987). This information provides the basis for further studies on the regulation of CSF-1 expression in the pig and its role in conceptus development.

Growth factors and cytokines are believed to play important roles in regulating cellular proliferation, differentiation and morphogenesis during mammalian embryonic development. They can function in an autocrine fashion within the embryo and/or in a paracrine fashion through interactions between the embryo and the uterus (Kaye PL, 1997; Kaye and Harvey, 1995; Schultz and Heyner, 1993). Some growth factors and cytokines have been demonstrated to be critical to implantation in mammals (Nie et al., 1997, Polan et al., 1995; Harvey et al., 1995). For example, a lack of endometrial expression of LIF could result in the failure of embryo implantation in the mouse (Stewart et al., 1992; Shellard et al., 1996). A number of growth factors and cytokines have been shown to be present in blastocysts and the uterus during the peri-implantation period in the pig, although little or no information exists at this point regarding their functions (Geisert and Yelich, 1997). The expression of CSF-1 at both the mRNA and protein level has been reported previously in porcine uterine, conceptus, allantochorionic and fetal tissues during the post-implantation stages of pregnancy by Tuo et al. (1995). In order to understand the role of CSF-1 during early embryonic development and implantation in the pig, the expression of CSF-1 and its receptor (c-fms) transcripts was determined in blastocysts and the endometrium during early gestation. In contrast to an

earlier study in which expression of c-fms but not CSF-1 was detected in pre-implantation embryos in the mouse (Arceci et al., 1992), expression of both c-fms and CSF-1 transcripts were detected in both blastocysts and the endometrium during the peri-implantation period in the present study. This result suggests that CSF-1 might play both paracrine and autocrine roles in embryonic development during early gestation in the pig. It has been demonstrated in mice and humans that CSF-1 is a growth factor involved in trophoblast proliferation and differentiation (Guilbert et al., 1991; Pollard et al., 1987). Therefore, we speculate that both endometrial and embryonic expression of CSF-1 could be directly involved in the proliferation and differentiation of porcine trophoblast cells, which is essential for the rapid expansion and elongation of blastocysts during pre-implantation development in the pig. A low level of c-fms expression in blastocysts prior to implantation might partially contribute to the non-invasiveness of porcine trophoblast cells during pregnancy.

The roles of MMPs and TIMPs in tissue remodeling during embryo implantation have been extensively studied in rodents and humans (Damsky et al., 1997; Bass et al., 1997; Alexander et al., 1996). A large amount of convincing evidence indicates that trophoblast-derived MMP-9 is responsible for the invasive type of embryo implantation seen in humans and rodents through its ability to degrade the basement membrane of the endometrium. In the second experiment of the present study, MMP-9 expression has not been detected by RT-PCR, which is in agreement with a previous study by Menino et al. (1997). These data indicate that a lack of MMP-9 expression by blastocysts during the peri-implantation period might partially explain the non-invasive type of implantation seen in pigs (Fig. 5.1). This observation provides further indirect

evidence for the essential role of MMP-9 in embryo implantation in mammals with invasive types of placentation (Salamonsen, 1999). Low levels of MMP-2 and MMP-9 transcripts were detected in Jag-1 trophoblast cells by RT-PCR, and significant levels of proMMP-2 and proMMP-9 were shown by zymography in the present study. Interestingly, Jag-1 cells were able to penetrate a layer of Matrigel artificial basement membrane in an *in vitro* invasion chamber model system, (Chai et al., unpublished observations). These observations provide an explanation at the molecular level for previous histological observations on the invasiveness of porcine embryonic trophoblast cells at ectopic sites (Samuel and Perry, 1972). The differential behavior of porcine trophoblast cells under *in vivo* and *in vitro* conditions suggests that negative regulatory factor (s), possibly present in the uterine fluid and/or induced within the blastocyst itself, might suppress the transcription of the MMP-9 gene in trophoblast cells of blastocysts.

Cell migration and interactions during early embryogenesis require the involvement of MMPs, as demonstrated in the mouse (Behrendtsen and Werb, 1997). In the second experiment of the present study, co-expression of MMP-2, TIMP-2 and MMP-14 transcripts were observed in blastocysts during the peri-implantation period. In addition, proMMP-2 levels in uterine flushings of pregnant gilts were elevated during this period. TIMP-2 expression was observed in all type of cells in porcine blastocysts at the time of embryo-uterine contact (Menino et al., 1997). Collectively, these results indicate that the MMP-2/TIMP-2/MMP-14 system might contribute to the spatially regulated extracellular proteolysis required for the extraembryonic mesoderm migration in porcine blastocysts (Fig. 5.1). This is consistent with the proposed role of this system in murine embryogenesis (Apte et al., 1997). Demonstrated expression of the MMP system in

porcine endometrium in our study suggests that extensive endometrial tissue remodeling occurs to prepare the endometrium for embryo implantation in the pig. The preliminary observations of the expression of other MMPs and MMP regulators, obtained in the present study, should pave the way for further examination of the functions of these factors during embryo development and implantation in the pig.

Mammalian early embryogenesis and implantation involve extensive cell-cell and cell-extracellular matrix interactions. These cellular interactions are known to be mediated by several classes of cell surface adhesion molecules, the most important of which are the integrins (Aplin, 1997; Lessey, 1997; Gille and Swerlick, 1996; Damsky et al., 1993; Hynes, 1992). Using indirect immunocytochemical techniques, some integrin proteins were previously localized in porcine uterine epithelium and trophoblast *in vivo* and *in vitro* by Bowen et al. (1996 and 1997). In order to understand the role of integrins during embryonic development in the pig, the third experiment of the present study was taken to examine the expression patterns of transcripts for eight integrin subunits (α1, α2, α3, α4, α5, αv, β1 and β3) in blastocysts and the endometrium during early gestation using an RT-PCR approach. Expression of α2, α3, αv, β1 and β3 transcripts in blastocysts correlates with the extraembryonic endodermal and mesodermal migration processes during early embryogenesis in the pig. Expression of α1, α2, α3, αv, β1 and β3 transcripts have been observed in Jag-1 cells and Day 28-placental tissue. These findings suggest important roles for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig. Based on the present data, in conjunction with previous studies, a hypothetical role of integrins during early

Table 5.1 Cellular regulation of integrin, MMPs and TIMPs expression by growth factors/cytokines

Experimental cell System	Gene Regulated	Regulator	Reference
Human dermal fibroblasts	α1 ↓; α5 ↑	PDGF	Gailit et al., 1996
Human breast and esophageal cancer cells	α2 ↑; α3 ↑	HBGF	Narita et al., 1996; Sato et al., 1996
Chick neuroepithelial cells	α6 ↑	IGF-I	Frade et al., 1996
Human endothelial cells	α1 ↑; α2 ↑	VEGF	Senger et al., 1997
Human salivary gland duct cells and rat mesangil cells	β1 ↑	TGF-β1	Azuma et al., 1996; Kagami et al., 1996
Human dermal fibroblasts	α1 ↑; α5 ↑	TNF-α; INF-γ; IL-1	Gailit et al., 1996
Human endometrial stromal cells	TIMP-1 ↑; TIMP-3 ↑	IL-1	Huang et al., 1998
Human normal and maligmant keratinocytes and ovarian cancer cells	MMP-9 ↑	EGF	Charvat et al., 1998; Elleroek et al., 1998
Human uterine cervical fibroblasts	MMP-3 ↑; TIMP-1 ↑	EGF	Hosono et al., 1996
Keratinocytes; kidney and mammary epithelial cells	TIMP-3 ↑	HGF	Castagnino et al., 1996
Lung cancer cells	MMP-2 ↑	IGF-I	Long et al., 1998
Human breast cancer cells	MMP-9 ↑	EGF; Amphiregulin	Kondapaka et al., 1997
Human prostatic cancer cells	MMP-2 ↓; TIMP-1 ↑	IL-4; IL-10	Wang et al., 1996
Human prostatic cancer cells	MMP-2 ↑; MMP-9 ↑	TGF-β1	Sehgal & Thompson et al., 1999
Human vascular smooth muscle cells	MMP-1 ↑; MMP-3 ↑; MMP-9 ↑	VEGF	Wang & Keiser, 1998

embryogenesis and implantation in the pig is shown schematically in Figure 5.2. The αv subunit was found on the apical surface of the uterine epithelium but not of the trophoblast during early pregnancy in the pig by Bowen et al. (1996). This suggests that endometrial integrins αvβ1 and αvβ3 might play a critical role in the attachment between the trophoblast and the uterine epithelium. Embryonic integrins αvβ1 and αvβ3 could instead facilitate the extraembryonic endodermal and mesodermal cell migration. A lack of strong expression of the β1 subunit in trophoblast cells in the pig is consistent with the critical role of β1 integrins in the highly invasive implantation seen in human and mouse.

Molecular interactions at the embryo-maternal interface during early pregnancy in mammals appear to be mediated through embryonic cytokines (Reviewed by Simon et al., 1998 and Martal et al., 1997). Harvey et al. (1995) showed that LIF and EGF could stimulate the secretion of uPA and MMP-9 in day-7 mouse blastocysts during their outgrowth *in vitro*. Recently, a number of growth factors/cytokines have been shown to regulate the expression and activity of integrins, MMPs and TIMPs in many types of normal and malignant cells (Table 5.1). As an example, IL-1β was shown to both increase the expression of MMP-9 and decrease the expression of TIMP-1 and TIMP-3 in human endometrial stromal cells (Huang et al., 1998). In contrast, the same authors also demonstrated that TGF-β augmented the expression of TIMP-1 and TIMP-3 but did not affect MMP-9 expression. As a result, it was of interest to know whether other growth factors/cytokines are expressed during early conceptus development in the pig. RT-PCR approaches have been used extensively in measuring the expression of cytokines in porcine immune cell populations (Dozois et al., 1997; Reddy et al., 1996; Vezina et al., 1995). Therefore, an additional experiment was carried out as a preliminary survey on the

expression of 9 cytokines, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, TNF-β and IFN-γ, during early conceptus development in the pig (Table 5.2 and Appendix A). Our preliminary data indicates that expression of IL-1, IL-6, IL-8 and IFN-γ is developmentally regulated in blastocysts during the peri-implantation period (Fig. 5.3). However, expression of IL-2, IL-4, IL-10, TNF-α and TNF-β was not observed in blastocysts at any of the developmental stages examined, or in the Jag-1 cells, or in Day 28 placental tissue (Fig. 5.3).

Interestingly, two IFN-γ cDNA fragments of 378 bp and 300 bp were amplified in both spherical blastocysts of 8-10 mm in diameter (Panel S3, Fig. 5.3) and all elongated blastocysts (Panels F1 to F4, Fig. 5.3), but not in the two earlier spherical stages (Panels S1 & S2, Fig. 5.3). This result is in agreement with previous studies by Ramsoondar (1994) and Lefevre et al. (1990). Both studies showed that two species of INF-γ mRNA with sizes of approximately 1. 4 kb and 1.3 kb, respectively, were present in Day 14 to 17 blastocsyts. It is likely that cDNA fragments of 378 bp and 300 bp were derived from IFN-γ mRNA species of 1. 4 kb and 1.3 kb, respectively. Furthermore, two species of IFN-γ mRNA exhibited different patterns of expression in blastocysts. Progressively increased expression was observed for the IFN-γ mRNA represented by the 378-bp cDNA fragment as blastocysts developed from spherical to elongated shapes (Panels S1 to F4, Fig. 5.3). In contrast, expression of the IFN-γ mRNA represented by the 300-bp cDNA fragment first appeared and was dominant in spherical blastocysts of 5-6 mm in diameter, and decreased as blastocyts elongated (Panels S3 to F4, Fig. 5.3). Expression of IFN-γ transcripts was not detected in Jag-1 cells (Panel T, Fig. 5.3),

although low expression of the IFN-γ mRNA represented by the 378-bp cDNA fragment was observed in Day-28 placental tissue (Panel P, Fig. 5.3).

Complex relationships between growth factors/cytokines, integrins, MMP/ TIMP and extracellular matrix have been realized during normal and abnormal cellular processes. For example, the interaction between epidermal growth factor (EGF) and integrin α3β1 has been shown to contribute to migratory and invasive properties of gliomas (Tysnes et al., 1997). Collaboration between platelet-derived growth factor (PDGF) receptor β and integrin α3β1 was shown to be required for the motile response of human mesothelioma cells to PDGF BB *in vitro* Klominek et al., 1998), as was the interaction between insulin-like growth factor-I (IGF-I) receptor and integrin αvβ3 necessary for the cellular response of vascular smooth muscle cells to IGF-I (Zheng and Clemmons, 1998). Furthermore, αvβ1 can function as a receptor for latent TGF-β, which might facilitate the role of latent TGF-β in the ECM (Munger et al., 1998). The interaction between latent TGF-β and αvβ1 may localize latent TGF-β to the surface of specific cells and allow the TGF-β gene product to initiate signals by both TGF-β receptor- and integrin-mediated pathways. In addition, a functional cooperation between IGF-I receptor and integrin αvβ5 was shown to be required for pulmonary metastasis of multiple tumor cell types *in vivo* (Brooks et al., 1997). Activation of both IGF-I receptor and integrin α5β1 is required for IGF-I to stimulate wound healing on rabbit ears (Galiano et al., 1996). A more intriguing observation is that the focal adhesion kinase p125 (FAK), a cytosolic tyrosine kinase, mediates signal transduction of not only integrins but also tyrosine kinase receptors including PDGF, HGF, insulin and IGF-I receptors (Baron et al., 1998). Therefore, as a member of the tyrosine kinase receptor

family, c-fms might be able to mediate cellular processes, alone or in collaboration with integrins and MMPs, during implantation and early embryogenesis in the pig. This hypothesis needs to be verified by further functional studies of porcine CSF-1. In relation to porcine implantation, an immediate study that could be carried out is to examine the role of CSF-1, IL-1, IL-6, IL-8 and IFN-γ in regulating the expression of trophoblastic integrins and/or MMPs and their regulators in Jag-1 cells.

MMP-2 and integrin $\alpha v\beta 3$ were co-localized on angiogenic blood vessels and melanoma cells *in vivo*, and expression of $\alpha v\beta 3$ on cultured melanoma cells enabled these cells to bind MMP-2 in a proteolytically active form, facilitating directed cellular invasion *in vitro* (Brooks et al., 1996). Antibodies against $\alpha 3$ and $\alpha 2$ integrin subunits could induce the activated form of MMP-2 and enhance proMMP-2 secretion and thereby facilitate cell invasion through reconstituted basement membrane (Matrigel) by human rhabdomyosarcoma cells *in vitro* (Kubota et al., 1997). Integrin $\alpha 2\beta 1$ has been shown to be a positive regulator of MMP-1 and type I collagen expression by osteogenic cell lines (Riikonen et al., 1995). Integrin ligation could regulate the expression of cytokine receptors on the cell surface of polymorphonuclear leukocytes (PMN) through signaling pathways involving protein tyrosine kinases ("outside-in" signalling), thereby influencing the chemotaxis of PMN into the interstitium (Simms et al., 1997; Hynes, 1992).

As our knowledge accumulates on the regulation and interactions between growth factors/cytokines, MMPs and integrins, it should be possible to decipher in detail the molecular mechanisms regulating implantation and early embryogenesis in the pig. As a result, approaches could be developed to minimize or prevent high embryonic loss

occurring during this period of development in order to improve further reproductive efficiency in this important domestic animal.

Figure 5.1 Schematic representation of the role of matrix matelloproteinase (MMP) system in implantation and early embryogenesis in the pig. The lack of expression of MMP-9 in the blastocyst and the co-localization of MMP-2 and TIMP-2 in the uterine lumen are consistent with the formation of the non-invasive type of placentation in the pig. The co-expression of the tri-molecular complex, MMP-2/TIMP-2/TIMP-14, in blastocysts would facilitate cell migration during mesodermal development in the pig.

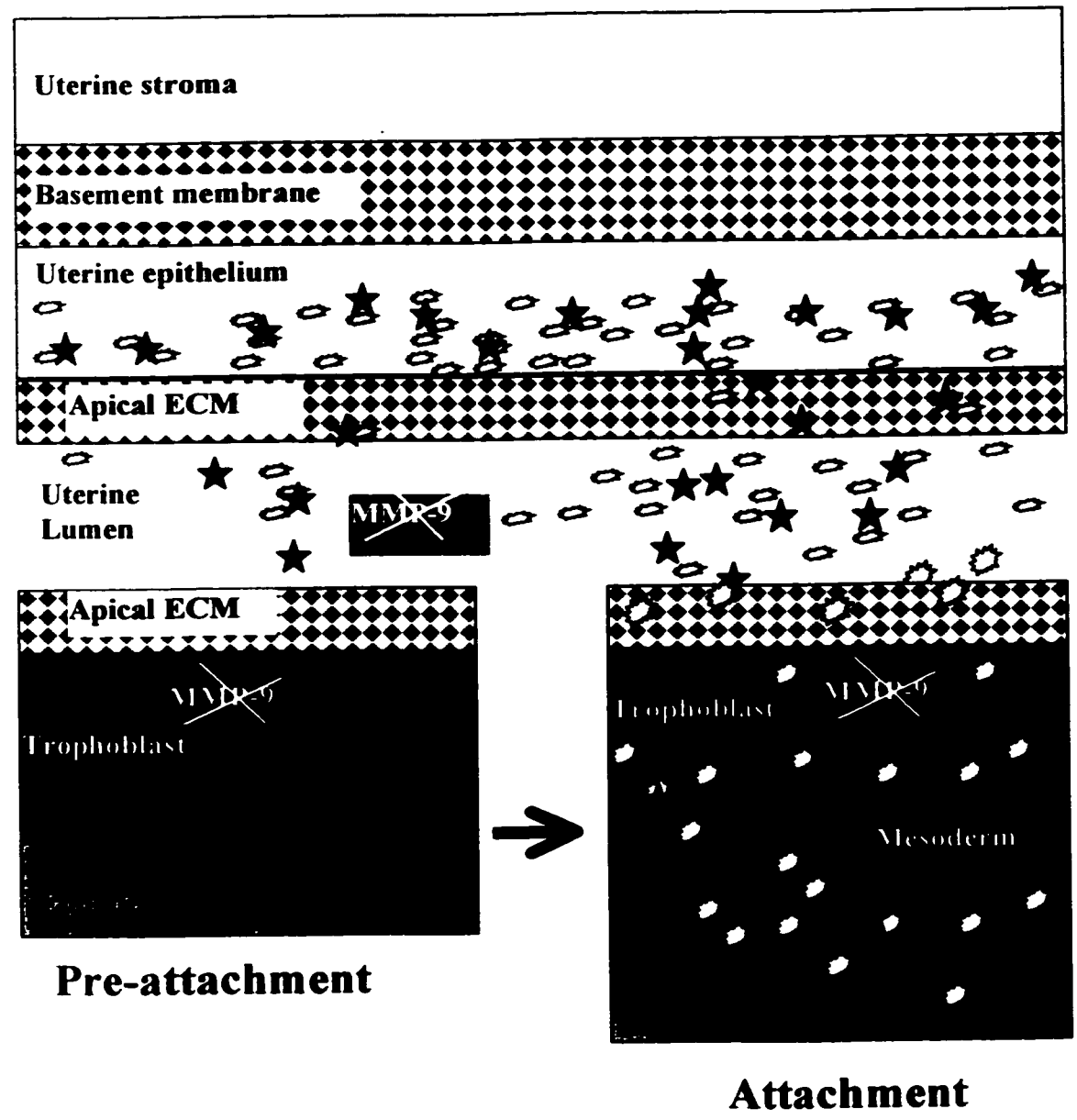

Uterine stroma
Basement membrane
Uterine epithelium
Apical ECM
Uterine
Lumen
MMP-9
Apical ECM
MMP-9
Trophoblast
Trophoblast
MMP-9
Mesoderm
Pre-attachment
Attachment
Type IV collagen
(Substrate of MMP-2)
Fibronectin
(Substrate of MMP-2)
MMP-2
TIMP-2
Vitronectin

Figure 5.2 Schematic representation of the hypothetical role of the integrin family in early embryogenesis and implantation in the pig. An absence of αv subunits at the apical surface of the trophoblast during early pregnancy suggests that endometrial integrins αvβ1 and αvβ3 might play a critical role in the attachment between the trophoblast and the uterine epithelium. Embryonic integrins αvβ1 and αvβ3 could facilitate the extraembryonic endodermal and mesodermal cell migration. A lack of strong expression of the β1 subunit in trophoblast cells in the pig with a non-invasive type of implantation is consistent with the critical role of β1 integrins in the invasive implantation seen in human and mouse.

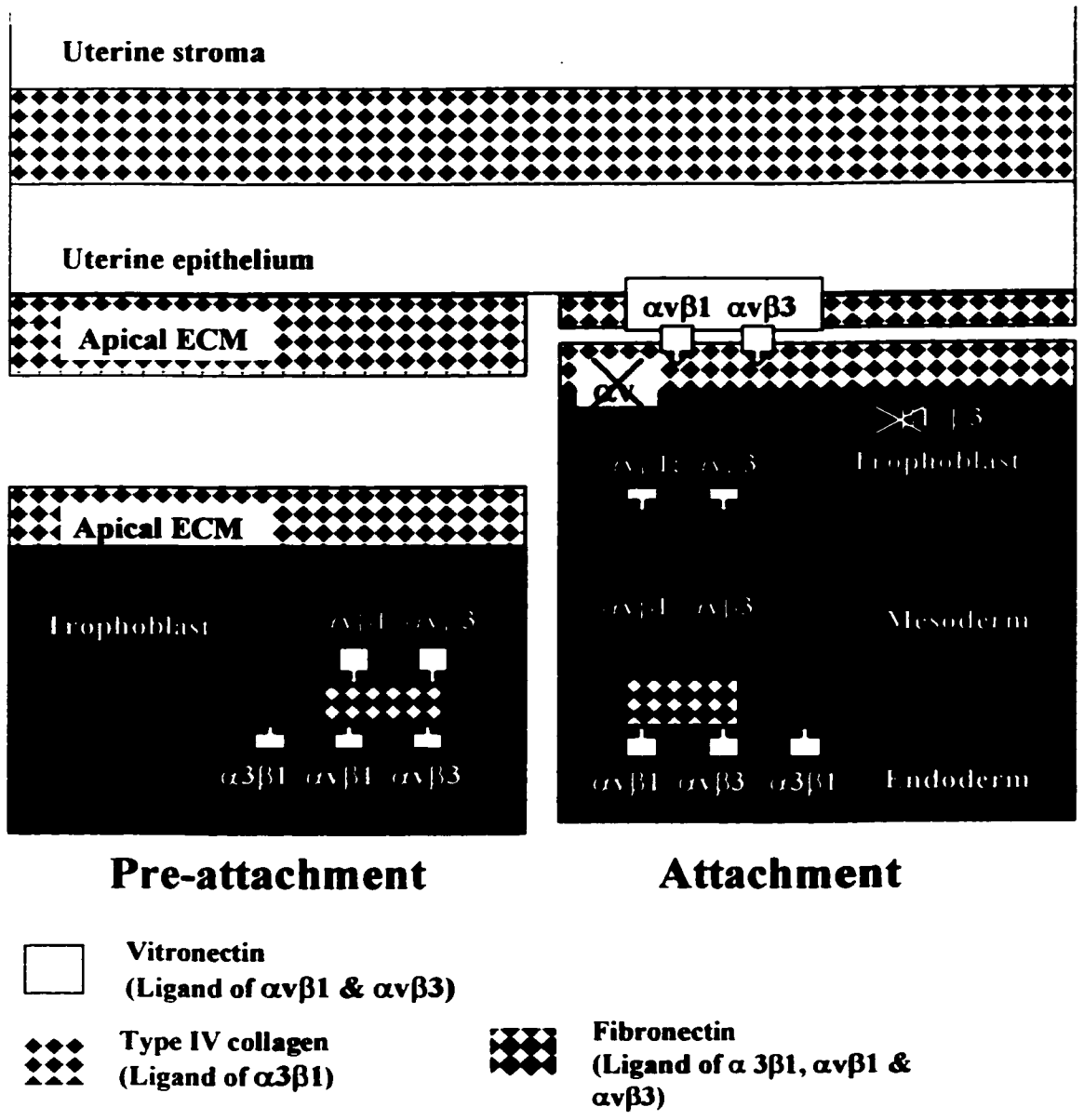
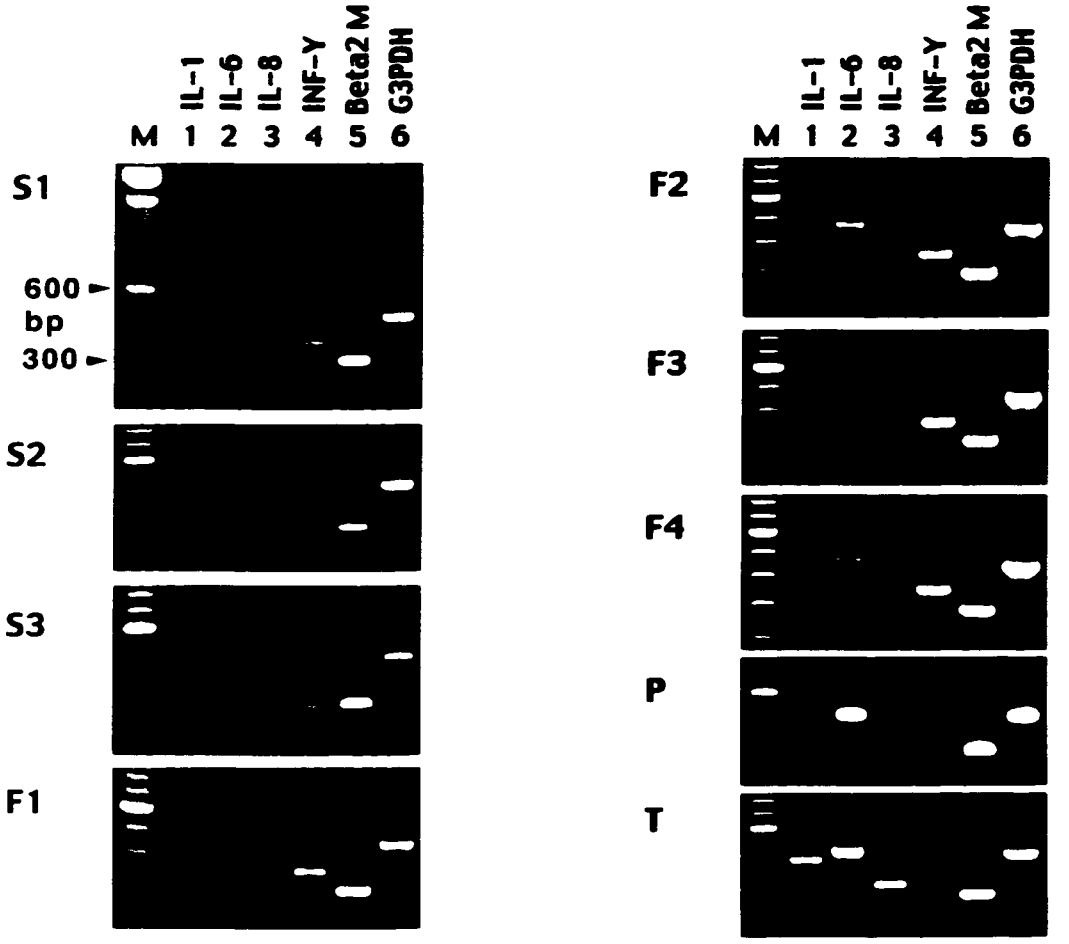


Figure 5.2 Schematic representation of the potential role of the integrin family in the pig.

Figure 5.3 Characterization of the expression for IL-1 (Lane 1), IL-6 (Lane 2), IL-8 (Lane 3), and IFN-γ (Lane 4) transcripts in blastocysts during the peri-implantation period in the pig. Expression of IL-2, IL-4, IL-10, IL-12, TNF-α and TNF-β transcripts was not detected at all stages of blastocyst development examined. M, 100-bp DNA ladder.


M
IL-1
IL-6
IL-8
INF-Y
Beta2 M
G3PDH
1
2
3
4
5
6
S1
600
bp
300
S2
S3
F1
F2
F3
F4
P
T

REFERENCES

Azuma M. Fukui K. Yuki T. Tamatani T. Motegi K. Hoque MO. Yoshida H. Sato M. Expression of integrin subunits in normal and malignant human salivary gland cell clones and its regulation by transforming growth factor-beta 1. Cancer Letters. 109(1-2):91-9, 1996

Baron V. Calleja V. Ferrari P. Alengrin F. Van Obberghen E. p125Fak focal adhesion kinase is a substrate for the insulin and insulin-like growth factor-I tyrosine kinase receptors. Journal of Biological Chemistry. 273(12):7162-8, 1998

Bowen JA. Bazer FW. Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biology of Reproduction. 55(5):1098-106, 1996

Brooks PC. Klemke RL. Schon S. Lewis JM. Schwartz MA. Cheresh DA. Insulin-like growth factor receptor cooperates with integrin alpha v beta 5 to promote tumor cell dissemination in vivo. Journal of Clinical Investigation. 99(6):1390-8, 1997

Brooks PC. Stromblad S. Sanders LC. von Schalscha TL. Aimes RT. Stetler-Stevenson WG. Quigley JP. Cheresh DA. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell. 85(5):683-93, 1996

Castagnino P. Soriano JV. Montesano R. Bottaro DP. Induction of tissue inhibitor of metalloproteinases-3 is a delayed early cellular response to hepatocyte growth factor. Oncogene. 17(4):481-92, 1998

Charvat S. Chignol MC. Souchier C. Le Griel C. Schmitt D. Serres M. Cell migration and MMP-9 secretion are increased by epidermal growth factor in HaCaT-ras transfected cells. Experimental Dermatology. 7(4):184-90, 1998

Dozois CM. Oswald E. Gautier N. Serthelon JP. Fairbrother JM. Oswald IP. A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression. Veterinary Immunology & Immunopathology. 58(3-4):287-300, 1997

Ellerbroek SM. Hudson LG. Stack MS. Proteinase requirements of epidermal growth factor-induced ovarian cancer cell invasion. International Journal of Cancer. 78(3):331-7, 1998

Fischer B. Platelet-derived growth factor-receptor-beta in pig and cattle blastocysts. Acta Anatomica. 159(4):194-203, 1997).

Frade JM. Marti E. Bovolenta P. Rodriguez-Pena MA. Perez-Garcia D. Rohrer H. Edgar D. Rodriguez-Tebar A. Insulin-like growth factor-I stimulates neurogenesis in chick retina by regulating expression of the alpha 6 integrin subunit. Development. 122(8):2497-506, 1996

Gailit J. Xu J. Bueller H. Clark RA. Platelet-derived growth factor and inflammatory cytokines have differential effects on the expression of integrins alpha 1 beta 1 and alpha 5 beta 1 by human dermal fibroblasts in vitro. Journal of Cellular Physiology. 169(2):281-9, 1996

Galiano RD. Zhao LL. Clemmons DR. Roth SI. Lin X. Mustoe TA. Interaction between the insulin-like growth factor family and the integrin receptor family in tissue repair processes. Evidence in a rabbit ear dermal ulcer model. Journal of Clinical Investigation. 98(11):2462-8, 1996

Guilbert LJ. Athanassakis I. Branch DR. Christopherson RJ. Crainie M. Garcia-Lloret M. Mogil R. Morrish D. Ramsoondar J. Vassiliadis S. Wegmann TG. The placenta as an immune-endocrine interface: placenta cells as targets for lympho-hematopoietic cytokine stimulation. In: Wegmann TG, Gill TJ, Nisbet-Brown (eds), Molecular and Cellular Immunobiology of the Maternal-Fetal interface. New York: Oxford University press, 1991. pp. 261-276.

Harvey MB. Leco KJ. Arcellana-Panlilio MY. Zhang X. Edwards DR. Schultz GA. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Development. 121(4):1005-14, 1995

Hosono T. Ito A. Sato T. Nagase H. Mori Y. Translational augmentation of pro-matrix metalloproteinase 3 (prostromelysin 1) and a tissue inhibitor of metalloproteinases (TIMP)-1 mRNAs by epidermal growth factor and transforming growth factor alpha, but not by interleukin 1 alpha or 12-O-tetradecanoylphorbol 13-acetate in human uterine cervical fibroblasts: the possible involvement of an atypical protein kinase C. Biological & Pharmaceutical Bulletin. 19(10):1285-90, 1996

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 69(1):11-25, 1992

Huang HY. Wen Y. Irwin JC. Kruessel JS. Soong YK. Polan ML. Cytokine-mediated regulation of 92-kilodalton type IV collagenase, tissue inhibitor or metalloproteinase-1 (TIMP-1), and TIMP-3 messenger ribonucleic acid expression in human endometrial stromal cells. Journal of Clinical Endocrinology & Metabolism. 83(5):1721-9, 1998

Kagami S. Kuhara T. Yasutomo K. Okada K. Loster K. Reutter W. Kuroda Y. Transforming growth factor-beta (TGF-beta) stimulates the expression of beta1 integrins and adhesion by rat mesangial cells. Experimental Cell Research. 229(1):1-6, 1996

Klominek J. Baskin B. Hauzenberger D. Platelet-derived growth factor (PDGF) BB acts as a chemoattractant for human malignant mesothelioma cells via PDGF receptor beta-integrin alpha3beta1 interaction. Clinical & Experimental Metastasis. 16(6):529-39, 1998

Kohka H. Yoshino T. Iwagaki H. Sakuma I. Tanimoto T. Matsuo Y. Kurimoto M. Orita K. Akagi T. Tanaka N. Interleukin-18/interferon-gamma-inducing factor, a novel cytokine, up-regulates ICAM-1 (CD54) expression in KG-1 cells. Journal of Leukocyte Biology. 64(4):519-27, 1998

Kondapaka SB. Fridman R. Reddy KB. Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. International Journal of Cancer. 70(6):722-6, 1997

Kubota S. Ito H. Ishibashi Y. Seyama Y. Anti-alpha3 integrin antibody induces the activated form of matrix metalloprotease-2 (MMP-2) with concomitant stimulation of invasion through matrigel by human rhabdomyosarcoma cells. International Journal of Cancer. 70(1):106-11, 1997

Lefevre F. Martinat-Botte F. Guillomot M. Zouari K. Charley B. La Bonnardiere C. Interferon-gamma gene and protein are spontaneously expressed by the porcine trophectoderm early in gestation. European Journal of Immunology. 20(11):2485-90, 1990

Long L. Navab R. Brodt P. Regulation of the Mr 72,000 type IV collagenase by the type I insulin-like growth factor receptor. Cancer Research. 58(15):3243-7, 1998

Martal J. Chene N. Camous S. Huynh L. Lantier F. Hermier P. L'Haridon R. Charpigny G. Charlier M. Chaouat G. Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN-tau and other cytokines in early pregnancy. Reproduction, Fertility, & Development. 9(3):355-80, 1997

Menino AR Jr. Hogan A. Schultz GA. Novak S. Dixon W. Foxcroft GH. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. Developmental Genetics. 21(1):68-74, 1997

Munger JS. Harpel JG. Giancotti FG. Rifkin DB. Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. Molecular Biology of the Cell. 9(9):2627-38, 1998

Narita T. Kawakami-Kimura N. Sato M. Matsuura N. Higashiyama S. Taniguchi N. Kannagi R. Alteration of integrins by heparin-binding EGF-like growth factor in human breast cancer cells. Oncology. 53(5):374-81, 1996

Nebe B. Sanftieben H. Pommerenke H. Peters A. Rychly J. Hepatocyte growth factor enables enhanced integrin-cytoskeleton linkage by affecting integrin expression in subconfluent epithelial cells. Experimental Cell Research. 243(2):263-73, 1998

Nie GY. Butt AR. Salamonsen LA. Findlay JK. Hormonal and non-hormonal agents at implantation as targets for contraception. Reproduction, Fertility, & Development. 9(1):65-76, 1997

Okamura H. Tsutsi H. Komatsu T. Yutsudo M. Hakura A. Tanimoto T. Torigoe K. Okura T. Nukada Y. Hattori K. et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature. 378(6552):88-91, 1995

Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB, Stanley ER (1987): Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature 330 (6147): 484-486.

Rajavashisth TB, Eng R, Shadduck RK, Waheed A, Ben-Avram CM, Shively JE, Lusis AJ (1987): Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc. Natl. Acad. Sci. USA 84: 1157-1161.

Ramsoondar J, Christopherson RJ, Guilbert LJ, Wegmann T (1993): A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biol. Reprod. 49: 681-694.

Ramsoondar J. (1994) The immunobiology of the porcine placenta (Ph.D. thesis). University of Alberta, Edmonton, Canada. pp. 226.

Reddy NR. Wilkie BN. Mallard BA. Construction of an internal control to quantitate multiple porcine cytokine mRNAs by RT-PCR. Biotechniques. 21(5):868-70, 872, 875, 1996

Riikonen T. Westermarck J. Koivisto L. Broberg A. Kahari VM. Heino J. Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. Journal of Biological Chemistry. 270(22):13548-52, 1995

Sato M. Narita T. Kawakami-Kimura N. Higashiyama S. Taniguchi N. Akiyama S. Hashimoto T. Manabe T. Kannagi R. Increased expression of integrins by heparin-

binding EGF like growth factor in human esophageal cancer cells. Cancer Letters. 102(1-2):183-91, 1996

Sehgal I. Thompson TC. Novel regulation of type IV collagenase (matrix metalloproteinase-9 and -2) activities by transforming growth factor-beta1 in human prostate cancer cell lines. Molecular Biology of the Cell. 10(2):407-16, 1999

Senger DR. Claffey KP. Benes JE. Perruzzi CA. Sergiou AP. Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proceedings of the National Academy of Sciences of the United States of America. 94(25):13612-7, 1997

Shellard J. Perreau J. Brulet P. Role of leukemia inhibitory factor during mammalian development. European Cytokine Network. 7(4):699-712, 1996

Simms HH. D'Amico R. Bland KI. Integrin stimulation regulates polymorphonuclear leukocytes inflammatory cytokine expression. Annals of Surgery. 225(6):757-63; discussion 763-5, 1997

Simon C. Moreno C. Remohi J. Pellicer A. Cytokines and embryo implantation. Journal of Reproductive Immunology. 39(1-2):117-31, 1998

Stewart CL. Kaspar P. Brunet LJ. Bhatt H. Gadi I. Kontgen F. Abbondanzo SJ . Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature. 359(6390):76-9, 1992

Trusolino L. Serini G. Cecchini G. Besati C. Ambesi-Impiombato FS. Marchisio PC. De Filippi R. Growth factor-dependent activation of alphavbeta3 integrin in normal epithelial cells: implications for tumor invasion. Journal of Cell Biology. 142(4):1145-56, 1998

Tysnes BB. Haugland HK. Bjerkvig R. Epidermal growth factor and laminin receptors contribute to migratory and invasive properties of gliomas. Invasion & Metastasis. 17(5):270-80, 1997

Vezina SA. Roberge D. Fournier M. Dea S. Oth D. Archambault D. Cloning of porcine cytokine-specific cDNAs and detection of porcine tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-1 beta gene expression by reverse transcription PCR and chemiluminescence hybridization. Clinical & Diagnostic Laboratory Immunology. 2(6):665-71, 1995

Wang H. Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circulation Research. 83(8):832-40, 1998

Wang M. Fudge K. Rhim JS. Stearns ME. Cytokine regulation of the matrix metalloproteinases and their inhibitors in human papillomavirus-18 transformed human prostatic tumor cell lines. Oncology Research. 8(7-8):303-15, 1996

Zheng B. Clemmons DR. Blocking ligand occupancy of the alphaVbeta3 integrin inhibits insulin-like growth factor I signaling in vascular smooth muscle cells. Proceedings of the National Academy of Sciences of the United States of America. 95(19):11217-22, 1998

APPENDIX

EXPRESSION OF CYTOKINES IN BLASTOCYSTS DURING PERI-IMPLANTATION DEVELOPMENT IN THE PIG

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, and two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of estrus (standing heat) with a vasectomized boar. At 16 and 24 hours after onset of estrus, gilts were artificially inseminated using fresh-pooled crossbred semen. All animals were handled in accordance with the Canadian Council on Animal Care (1993). Pregnant gilts were slaughtered, and the reproductive tracts were recovered within 10 minutes of death.

B. Collection of Blastocysts, Placental Tissues and Jag-1 cells

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed briefly in physiological saline to remove uterine debris. Spherical blastocysts were classified according to their sizes into three groups: 1-2 mm (n=37), 5-6 mm (n=19) and 8-10mm (n=8) in diameter, which were collected from gilts on Days 9 (n=3), 10 (n=2) and 10.5 (n=1) of pregnancy, respectively (Table 4.1). The number of elongated blastocysts obtained from each pig was estimated by counting the free bulbous ends of aggregated blastocysts. Sections of placental tissue were obtained from the allantochorial membrane distal to the embryonic region from Day 28 conceptuses. A porcine

trophoblast cell line, Jag-1, was included for comparison of gene expression, and Jag-1 cells were cultured and harvested as described previously by Ramsoondar et al. (1993). All tissue and cell samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues or Jag-1 cells. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

D. Primers and RT-PCR

PCR Primers for cytokines and β2-microglobulin were from a previous study by Reddy et al. (1996), and primers for G3PDH (Lane 6) were synthesized as described by Yelich et al. (1997) (Table 6.1).

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 μg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 μl, containing 5 μM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% (v/v) Tween 20. Aliquots from the same RT reaction were used to amplify each of the target genes (Table 6.1) for each stage of blastocyst development. A master reaction mixture was prepared for PCR amplification of all target genes in that development stage. Therefore, PCR amplification of all target genes for each stage was

carried out under the same conditions, except that 0.4 µl of cDNA template were used for β2-microglobulin and G3PDH while 10 µl of cDNA template were used for all cytokines. PCR was performed with an initial denaturation step at 94 °C for 2 min, followed by 38 cycles (denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 min), and a final extension step at 72 °C for 7 min. All PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 Thermocycler in a reaction volume of 50 µl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 µM of each specific primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes.

REFERENCES

Ramsoondar J, Christopherson RJ, Guilbert LJ, Wegmann T (1993): A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biol. Reprod. 49: 681-694.

Reddy NR. Wilkie BN. Mallard BA. Construction of an internal control to quantitate multiple porcine cytokine mRNAs by RT-PCR. Biotechniques. 21(5):868-70, 872, 875, 1996

Yelich JV. Pomp D. Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biology of Reproduction 57:1256-65, 1997

Table 6.1 Primers for cytokines and predicted sizes of PCR products

Target genes	Primer sequences	Sizes of PCR products (bp)	cDNA amount applied for PCR
IL-1	CAGCTATGAGCCACTTCCTG(f) GTCACAGGAAGTTGCGAATC(r)	434	10 µl
IL-2	CAGTAACCTCAACTCCTGCCAC CATCCTGGAGAGATCAGCATTC	199	10 µl
IL-4	GTCTCACATCGTCAGTGC TCATGCACAGAACAGGTC	359	10 µl
IL-6	GGAACGCCTGGAAGAAGATG ATCCACTCGTTCTGTGACTG	470	10 µl
IL-8	TGCAGCTTCATGGACCAG GTACAACCTTCTTCTGCACC	323	10 µl
IL-10	GCTCTATTGCCTGATCTTCC GCACTCTTCACCTCCTCCAC	404	10 µl
TNF-α	CACTGAGAGCATGATCCGAG GGCTGATGGTGTGAGTGAGG	463	10 µl
TNF-β	ATGACACCACCTGGACG TGGTACACAGAGCGCAC	502	10 µl
IFN-γ	TGTACCTAATGGTGGACCTC TCTCTGGCCTTGGAACATAG	378	10 µl
β2 micro-globulin	CTGCTCTCACTGRCTGG ATCGAGAGTCACGTGCT	284	0.4 µl
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	452	0.4 µl