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The Expression of the Subtilisin-Like Proprotein Convertase SPC6 in the Decidua During Mouse Embryo Implantation

by

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ABSTRACT

The process of mouse embryo implantation is regulated and coordinated by many molecular events. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in the tissue remodeling process that occurs at the site of implantation. A dramatic increase of subtilisin-like proprotein convertase-6 (SPC6) transcripts was demonstrated at the onset of implantation. SPC6 expression exhibits a great degree of temporal and spatial overlap with TIMP3 expression. Transforming growth factor-beta1 (TGF-β1) can induce TIMP3 expression *in vitro* and both TGF-β1 and -β2 gene expression were detected in the decidua. The proposed hypothesis states that SPC6 may exert its effect through the activation of TGF-β, which may influence TIMP expression during the tissue remodeling process in embryo implantation. The results indicate that although attempts to inhibit SPC6 activity had no effect on TIMP3 mRNA levels in endometrial stromal cell cultures, the temporal and spatial expression of SPC6 in the decidua implies an important, yet undetermined, role in the implantation process.

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I would like to express my heartfelt thanks to my family and to my many friends for their constant love and support. I am forever indebted to my mother, who made many selfless sacrifices to ensure that her children received proper education and a moral upbringing. Thanks, Mom.

DEDICATION

To my family,

Gloria and Shing Keung Wong,

Kitty Wong-Honeychurch and Shaun Honeychurch,

and

Sam Wong.

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LIST OF ABBREVIATIONS

Units of Measure

°C	degrees centigrade
bp	base pair
cpm	counts per minute
g	gram
h	hour
kb	kilobase
kDa	kilodalton
kg	kilogram
L	litre
μ*Ci*	microCurie
μg	microgram
μl	microlitre
μm	micrometre
mg	milligram
ml	millilitre
mmol	millimole
μM	micromolar
mM	millimolar
M	molar
ng	nanogram
nM	nanomolar
V	volts

Chemical Compounds / Buffers / Solutions

BCIP	5-bromo-4-chloro-3-indoyl phosphate
DEPC	diethylpyrocarbonate

dH_2O	deionized distilled water
DMEM:F12	Dulbecco's modified Eagle's medium:F12
DMF	*N,N*-dimethylformamide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NBT	nitro blue tetrazolium salt
PBS(T)	phosphate buffered saline (tween-20)
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TBS(T)	Tris-buffered saline (tween-20)
TE	Tris EDTA buffer
Tris	tris(hydroxymethyl)aminomethane

Nucleic Acids

18S	18S ribosomal ribonucleic acid
28S	28S ribosomal ribonucleic acid
ATP	adenosine 5'-triphosphate
cDNA	complementary deoxyribonucleic acid
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate

GTP	guanosine 5'-triphosphate
mRNA	messenger ribonucleic acid
NTP	nucleoside 5'-triphosphate
RNA	ribonucleic acid
rRNA	ribosomal RNA
UTP	uridine 5'-triphosphate

General

α1-AT	alpha-1 anti-trypsin
α1-PDX	alpha-1 anti-trypsin Portland
BMP	bone morphogenetic protein
DIG	digoxigenin
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HRP	horse radish peroxidase
IGF	insulin-like growth factor
IS	implantation site
MMP	matrix metalloproteinase
NIS	non-implantation site
PACE	paired amino acid converting enzyme
pBS	Bluescript plasmid
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Rnase	ribonuclease
RT	reverse transcription
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPC	subtilisin-like proprotein convertase
Taq polymerase	*Thermus aquaticus* DNA polymerase
TGF-β	transforming growth factor-beta

TGN	*trans*-Golgi networks
TIMP	tissue inhibitors of metalloproteinases
T_m	melting temperature
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

1. Introduction

Implantation is the process by which a mammalian embryo attaches to the uterus of the mother and invades the uterine epithelium to initiate establishment of the placenta (reviewed Schultz and Edwards, 1997; Rinkenberger *et al.*, 1997). Often, spontaneous abortion of the embryo is the result of improper attachment and implantation. Failure of implantation is pertinent to *in vitro* fertilization programs, to diseases of pregnancy such as preeclampsia, and to the agricultural industry in which embryo loss associated with livestock species has economic consequences for producers.

Implantation is a complex process regulated and coordinated by many cellular and molecular events. By understanding some of the molecular components associated with this process, we might gain insight into clinically relevant issues such as infertility and chronic pregnancy failures. The aim of the research described in this thesis is to characterize the expression, control and function of a member of the subtilisin-like proprotein convertase (SPC) family, SPC6, during the implantation process. Because SPC6 may be involved in embryo implantation (Rancourt and Rancourt, 1997), an invasive process, this gene may also be implicated in the invasive properties of metastatic cancers. For the experiments described herein, the mouse is used as the animal model because both murine and human implantation are highly invasive processes that lead to the formation of a hemochorial placenta.

Subtilisin-like prohormone convertases or SPCs have been found to activate members of the matrix metalloproteinase (MMP) and transforming

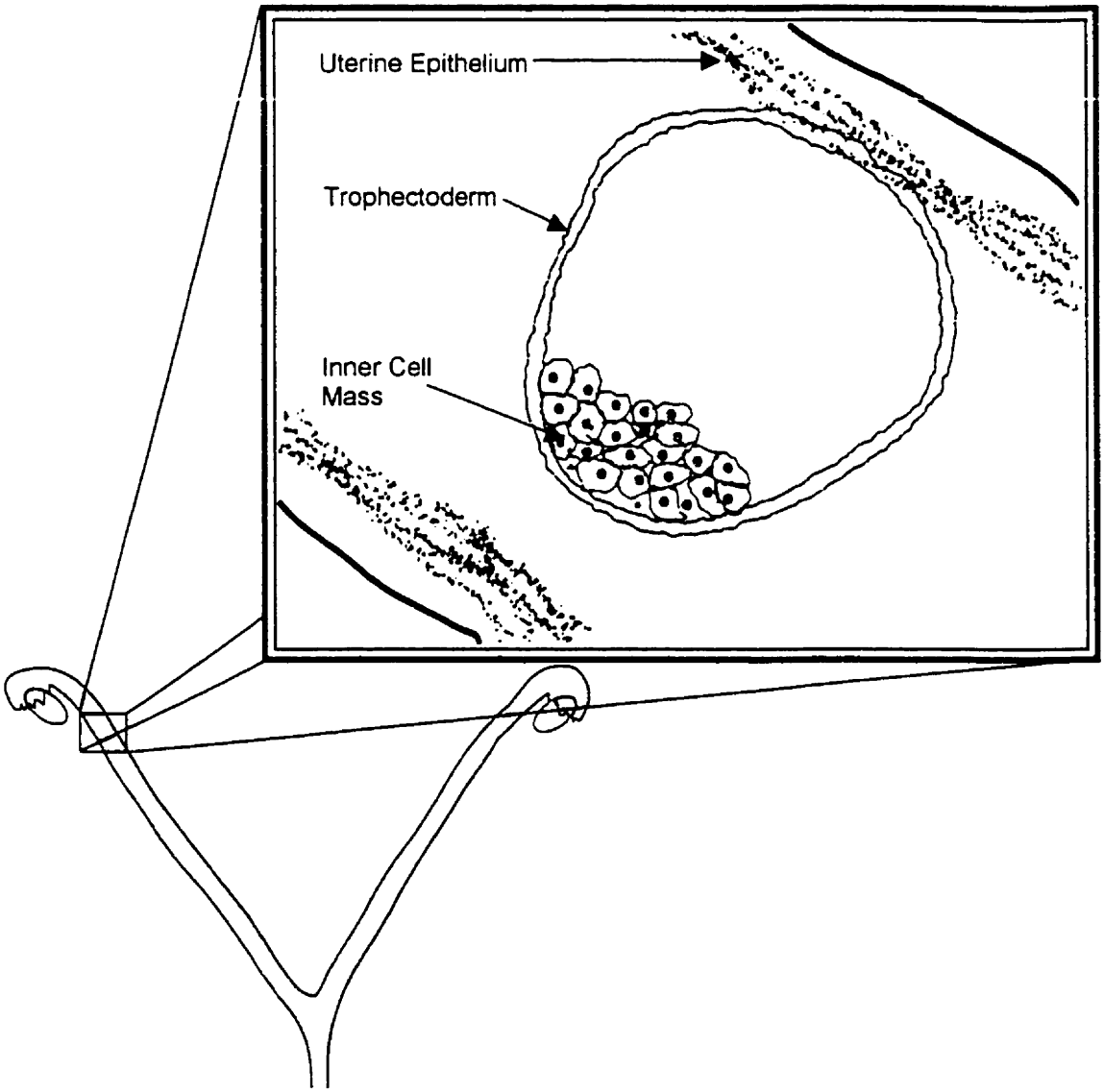
growth factor-beta (TGF-β) families (reviewed in Seidah and Chretien, 1997; Zhou *et al.*, 1999). In mammals, MMPs and their inhibitors (TIMPs) are required for the initiation and the control of tissue remodeling at the site of embryonic implantation as they are thought to mediate the invasiveness of the trophoblast of the embryo (Schultz and Edwards, 1997; Rinkenberger, *et al.*, 1997). Previous studies on the expression of the SPC6 gene showed transcript localized in the peri-implantation zone of the early, implanted embryo (6.5 days after fertilization) (Rancourt and Rancourt, 1997). The same study revealed a great degree of overlap between sites of expression of the SPC6 gene and the TIMP-3 gene. The hypothesis underlying the research in this thesis is that SPC6 may exert its influence through the activation of TGF-β to regulate the expression of MMPs and TIMPs, thereby facilitating the tissue remodeling process during mouse embryo implantation.

1.1 Implantation

Implantation is the process by which the developing embryo attaches to the uterus of the mother for the development of the placenta to provide the vascular connections for the transport of nutrients and the removal of waste (Schultz and Edwards, 1997; Rinkenberger *et al.*, 1997). After fertilization, the pre-implantation blastocyst migrates from the oviduct into the uterus to its point of attachment (Figure 1) and the embryo hatches from the zona pellucida by a combination of rhythmic contractions of the blastocyst and the production of strypsin by the mural trophectoderm that erodes the zona pellucida (Perona and

Figure 1: Onset of Blastocyst Implantation

The implantation process is initiated at day 4.5 post-coitum, after the embryo has proteolytically shed its zona pellucida. The sticky embryo secrets extracellular matrix degrading proteinases from the trophectoderm and the maternal endometrium responds by secreting inhibitors to ensure that the invasion is precise and limited.


Uterine Epithelium
Trophectoderm
Inner Cell
Mass

Wassarman, 1986). The process of attachment of the embryo to the uterus occurs at gestation days 4.5-5 (Rinkenberger *et al.*, 1997). During the early stages of implantation process, the embryo utilizes nutrients within the fluids in the maternal reproductive tract but upon completion of implantation at day 9 of gestation, a specialized and vascularized region, the placenta, is developed. Death and resorption of the mouse embryo occurs as a result of failure to implant or the inability to establish a functional placenta. This complex sequence of events is orchestrated by a variety of essential adhesion, signaling, and transcriptional regulatory molecules, and by proteins needed for cell cycle regulation, DNA repair and replication. The overall process of early pregnancy, in turn, is coordinated by a series of hormone signals that prepare the uterus for embryo implantation. Although the exact mechanism of how hormones regulate these events is unclear, it is known that hormones are responsible for the activation of proteins implicated in increasing uterine vascular permeability, prior to and at the time of blastocyst attachment.

Mammalian placentation is classified into three types, epitheliochorial, endotheliochorial and hemochorial, according to the degree of invasiveness into the uterus (Johnson and Everitt, 1980; Schultz and Edwards, 1997). Rodents and humans exhibit the hemochorial type, or the most invasive of all three types (Figure 2). When the invasive embryo contacts the maternal epithelium, the stromal tissues underlying the epithelium are rapidly affected within an hour of embryo-uterine contact, marking the beginning of the decidualization process (Johnson and Everitt, 1980). Furthermore, this effect is distributed over an

Figure 2: Examples of the Different Degrees of Invasiveness Between Primate, Rodent, and Ruminant Implantation.

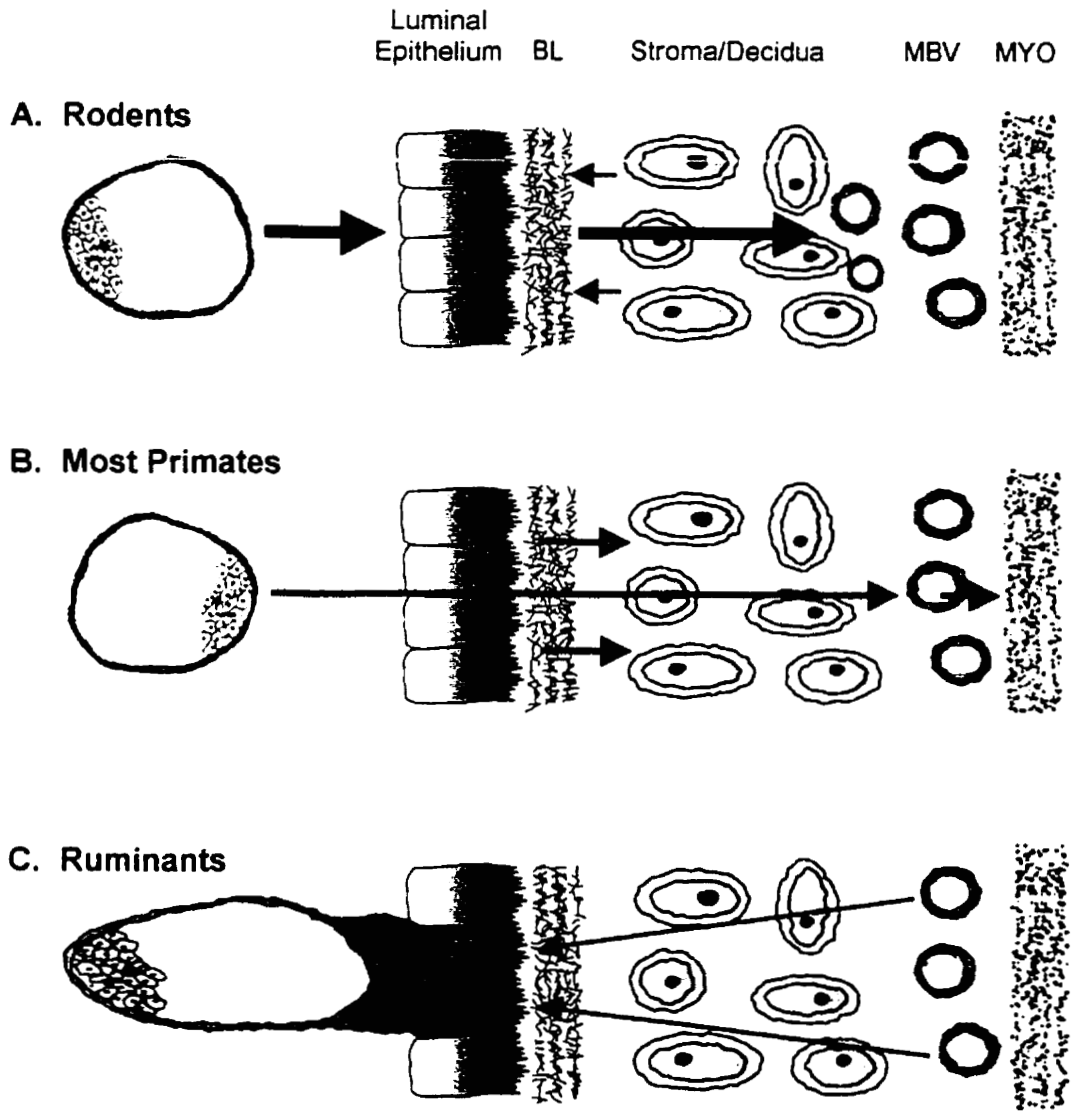
Among different species, the extent to which the trophoblast invade and penetrates the luminal epithelium and endometrium to establish contact with the maternal blood supply vary considerably.

A) In rodents, there is apoptosis of the uterine epithelial cells at the site of blastocyst apposition. The decidual cells then penetrate the basal lamina, and subsequently, a tightly constrained invasion within the endometrium by the trophoblast occurs.

B) The process of implantation in primates involves the trophectoderm passing through the epithelial cells, spreading across and eroding the basal lamina and eventually, invading through the stroma/decidua to the blood vessels deep in the endometrium.

C) In ruminants, there is little direct invasion of the trophoblast into the uterine stroma. Instead, a syncytium is formed by the trophoectoderm fusing with the luminal epithelial cells. There is no further invasion of the embryo and the syncytial cells penetrate the basal lamina at intervals. Nutrient is supplied by considerable development of the maternal blood vessels towards the syncytial layer.

Abbreviations: MBV - maternal blood vessels, MYO - myometrium, BL - basal lamina. (Modified from Salamonsen, 1999)

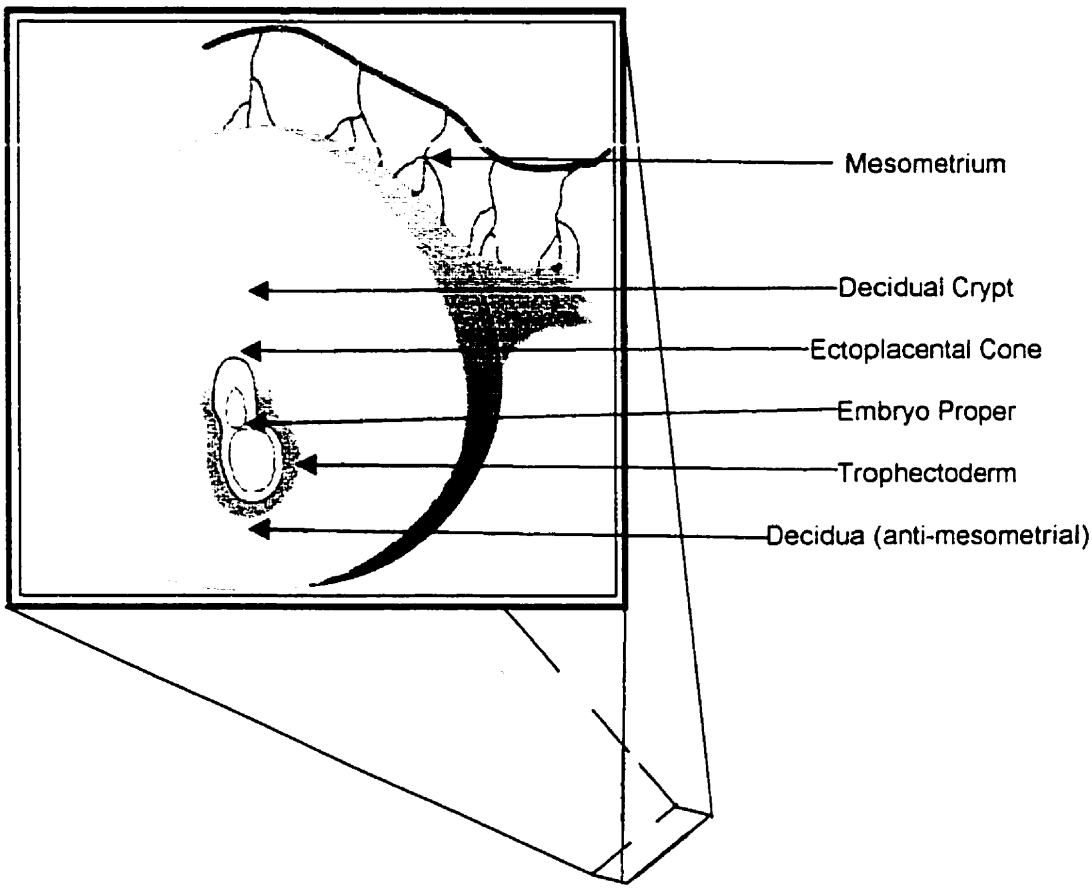

Luminal
Epithelium
BL
Stroma/Decidua
MBV
MYO
A. Rodents
B. Most Primates
C. Ruminants

extensive area of the endometrium. In the process of decidualization, one of the earliest visible responses of a receptive uterus is increased vascular permeability in the stromal tissue underlying the embryo. Vascular permeability is followed by localized swelling and compositional changes in the intercellular matrix, alterations in the morphology of the stromal cells and a progressive remodeling of capillary networks for sustaining the embryo through the early stages of gestation until the placenta has formed (Figure 3). In the highly invasive, hemochorial type of implantation, the embryo erodes the epithelium, stromal cells, connective tissues, and even the maternal blood vessels. The stromal cells of the uterus impede this event by developing protective mechanisms which restrict and control the depth of the invasion.

The invasiveness of the embryo is mediated by proteinases that are secreted by the trophoblast giant cells (see Salamonsen [1999] for a list of proteases and inhibitors produced by the conceptus and the maternal endometrium). Some proteases are required for the activation of regulatory molecules, while three families of proteases are involved in the remodeling or degradation of the extracellular matrix during implantation. Examples of proteinases that are produced by the implanting embryo include urokinase-type plasminogen activator (uPA) and its receptor, gelatinaseA/MMP-2, gelatinase B/MMP-9, and many others. Although rapid progress is being made in identifying the genes and cellular processes of implantation, the cascade of molecular events of many proteinases and their activating molecules has yet to be elucidated.

Figure 3: Cross-Section Through a Decidual Crypt

In response to the invasive embryo, the maternal stromal cells undergo decidualization and differentiate into decidual cells. After the embryo has properly implanted (day 6.5 post-coitum), it becomes encased in a decidual crypt. It is here, buried in the endometrium, that the embryo will grow and nourish on nutrients supplied through the placenta by the maternal blood vessels.


Mesometrium
Decidual Crypt
Ectoplacental Cone
Embryo Proper
Trophectoderm
Decidua (anti-mesometrial)

1.2 Proteinases Involved in Tissue Remodeling

There are three categories of proteinases that play a role in extracellular matrix (ECM) degradation (Salamonsen, 1999). Briefly, the three families are cysteine, serine and metallo-proteinases. Cysteine proteinases, like cathepsin B and cathepsin L, are lysosomal enzymes that act at an acidic pH. The cysteine proteinases are controlled by members of the cystatin superfamily which include both intracellular and extracellular forms. Increased production and secretion of cysteine proteinases and aberrant regulation of extracellular cystatin have been associated with the invasive phenotype of many metastatic cell types. Therefore, cysteine proteinases have also been postulated to play a role in embryo implantation. Cystatin C and cathepsins B and L have been shown to be expressed during mouse implantation and placentation. High doses of E-64, an inhibitor of cathepsin B and L, cause abnormal uterine decidualization and embryo development (Alfonso *et al.*, 1996).

Serine proteinases act mostly at neutral pH and are the largest class of mammalian proteinases. Many of this type of protease indirectly exert their action on matrix degradation by proteolytic activation of matrix metalloproteinases. Examples of serine proteinases include urokinase-type plasminogen activator (uPA), produced in the uterus, and kallikrein, produced mostly by endometrial fibroblasts. The action of these enzymes is balanced by specific inhibitors such as plasminogen activator inhibitor-1 (PAI-1), PAI-2, and the protease nexin, which limit the action of plasminogen activators (Vassali, *et al.*, 1991). The activity is focused to the vicinity of the cell by the cell surface

binding sites for uPA and plasminogen that are present on cell membranes (PAR). The proteolytic potential of the system is enhanced considerably by binding to these receptors (PAR). uPA and PAI expression are controlled by transcriptional mechanisms, driven by hormones and growth factors in a cell type-specific manner and this PA-PAI-PAR-plasmin system is thought to be a key player in the balance of matrix turnover (for a detailed review, see Salamonsen, 1999). Detailed studies of uPA have shown that it is expressed by trophoblast cells and their derivatives in implanting mouse embryos (Strickland *et al.*, 1976; Sappino *et al.*, 1991; Harvey *et al.*, 1995). Inhibition of its activity decreases the extent of trophoblast attachment and outgrowth *in vitro* (Kubo *et al.*, 1981).

The most critical enzymes for matrix degradation and remodeling belong to the third class of proteinases, the matrix metalloproteinases (MMPs). The processes of embryonic development, morphogenesis, reproduction, tissue resorption and tissue remodeling require the breakdown of the ECM in a well-coordinated and precise manner. In addition, roles for MMPs in tumor metastasis and invasion are well-documented (Toi *et al.*, 1998; Salamonsen, 1999; Nagase and Woessner, 1999; Kugler, 1999). MMPs are powerful proteinases whose substrate activities collectively allow them to degrade virtually all the components of both interstitial matrix and basement membranes. Production of the MMPs is transcriptionally regulated by growth factors, hormones, cytokines, and cellular transformation. All MMPs are synthesized as prepro-enzymes, and in most cases, secreted as inactive pro-MMPs. Once secreted, proteolytic cleavage of the precursor form is required for activation from

the latent protein to its active form. Once activated, MMPs are not necessarily available for tissue degradation because inhibition by endogenous inhibitors, α-macroglobulins, and tissues inhibitors of metalloproteinases (TIMPs) control precisely the proteolytic activities of MMPs by binding their active forms in a 1:1 stoichiometric ratio. Therefore, at any point of expression of MMP within a tissue, MMP-catalysed proteolysis of matrix will not occur unless active MMP is in molar excess over the inhibitors. In short, MMP production and proteolysis of cellular matrix are regulated both transcriptionally, by hormones and growth factors, and post-transcriptionally, by endogenous inhibitors such as TIMPs .

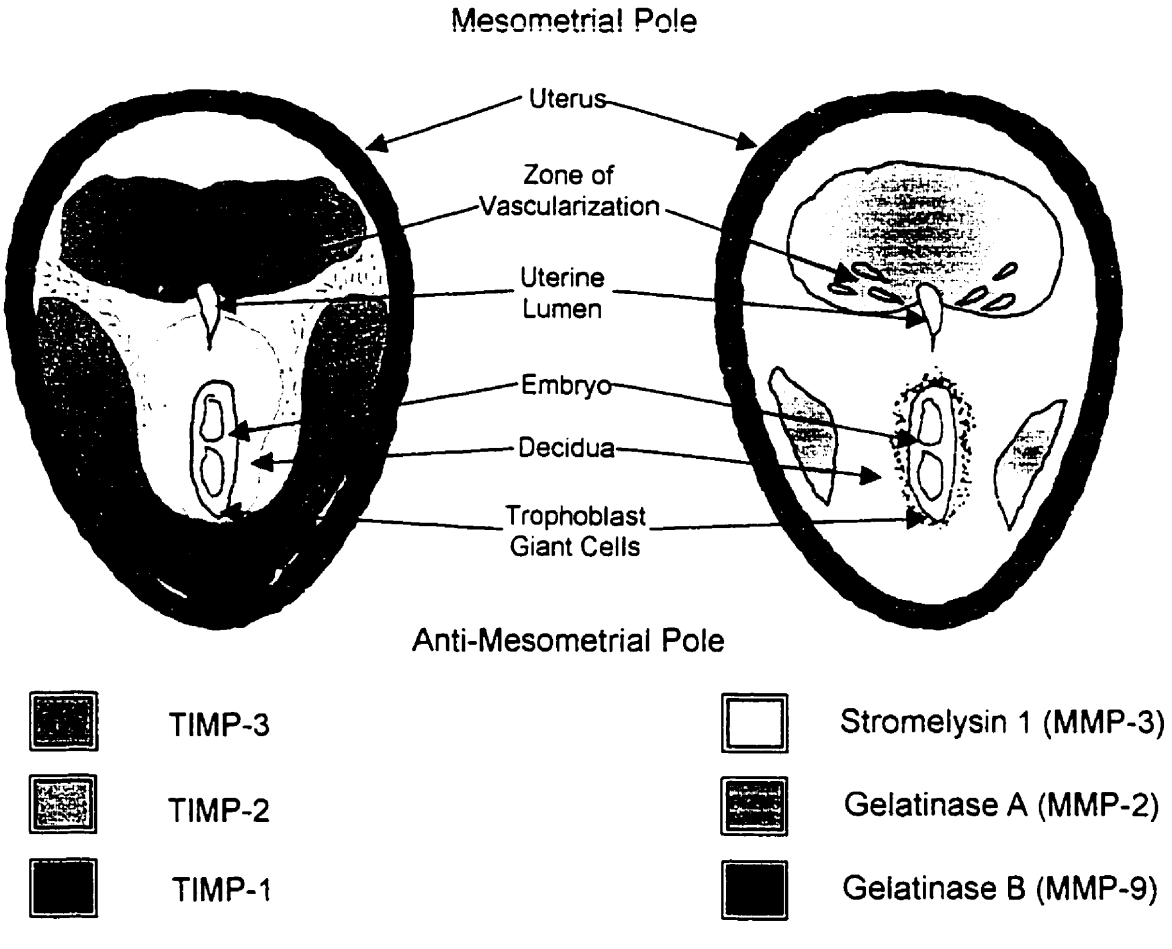
The major endogenous regulators of MMP activities are the tissue inhibitors of metalloproteinases (TIMP) proteins. There are four homologous TIMPs identified to date, TIMPs-1 to -4 that range in size from 21-30 kDa (Edwards *et al.*, 1997). TIMPs inhibit cell invasion *in vitro*, tumorigenesis and metastasis *in vivo*, angiogenesis *in vitro* and *in vivo*, and exhibit several additional biological functions independent of MMP activity (Leco *et al.*, 1994; Leco *et al.*, 1997; Salamonsen, 1999; Nagase and Woessner, 1999; Kugler, 1999). TIMPs inhibit MMP activity by binding to the catalytic domain of MMP in a 1:1 molar ratio. Recently, Gomis-Ruth *et al.* (1997) characterized the crystal structure of the complex formed between TIMP-1 and the catalytic site of MMP-3. In support of the concept that the local balance between the activities of MMPs and TIMPs is critical in determining the rate of ECM turnover, processes such as wound healing, ovulation, embryo implantation and mammary gland involution are facilitated by carefully coordinated changes in the production of MMPs and

TIMPs. Disruption of this delicate balance is implicated in pathological tissue damage and other disorders.

Because MMPs and TIMPs are extensively involved in tissue remodeling and the breakdown of the ECM, they have been the subject of intense study in the highly invasive process of implantation (Harvey *et al.*, 1995; Leco *et al.*, 1996; Alexander *et al.*, 1996; Das *et al.*, 1997; Salamonsen, 1999). During mouse embryo implantation, there is a very distinct and cell-specific expression pattern of MMP-2 and MMP-9, type IV collagenases, and TIMP-3. Das *et al.* (1997) found that an induction of transcription of MMP-2 occurs on day 3 in the stromal cells adjacent to the luminal epithelium, and this expression continues up to day 5 post-coitum. MMP-2 transcripts are not detected in the primary decidual zone on day 6 and on day 8, they are only found in the mesometrial pole where neovascularization occurs. In the uterus, MMP-9 mRNA is detected at low levels in some populations of stromal cells at the site of blastocyst apposition but its predominant expression is within trophoblast giant cells surrounding the embryo from day 6.5 to 8.5 of gestation (Harvey *et al.*, 1995; Leco *et al.*, 1996; Alexander *et al.*, 1996; Das *et al.*, 1997). Simultaneously, there are drastic changes in TIMP-3 expression. Elevated TIMP-3 expression is detected from day 6 to day 7 of pregnancy in maternal decidual cells proximal to the invading embryo, with intensity of staining decreasing by day 8.5 (Figure 4; Leco *et al.*, 1996; Alexander *et al.*, 1996). The coordinate expression of MMP-9 in trophoblast giant cells and TIMP-3 in maternal decidual cells surrounding the embryo from day 5.5 to 8.5 suggests a crucial role for each in the implantation-invasion process. Indeed, an

Figure 4: Spatial Expression Pattern of Various MMPs and TIMPs

The schematic representation of the localization of MMP and TIMP mRNAs in the mouse embryo implantation site on day 7.5 post-coitum. (Modified from Das *et al.*, 1997)


Mesometrial Pole
Uterus
Zone of
Vascularization
Uterine
Lumen
Embryo
Decidua
Trophoblast
Giant Cells
Anti-Mesometrial Pole
TIMP-3
TIMP-2
TIMP-1
Stromelysin 1 (MMP-3)
Gelatinase A (MMP-2)
Gelatinase B (MMP-9)

MMP inhibitor causes reduction in decidual size and embryo displacement when administered to pregnant mice during the implantation stage (Alexander *et al.*, 1996).

Presently, little is known about how TIMP-3 and MMP-9 expression are directly or indirectly regulated, although *in vitro* studies have demonstrated that leukemia inhibitory factor (LIF) and epidermal growth factor (EGF) can regulate proteinase activity in peri-implantation stage mouse embryos (Harvey *et al.*, 1995). Other studies have shown that TGF-β1 (Tamada *et al.*, 1990), TGF-β2 (Das *et al.*, 1992) and the TGF-β Type II receptor (Roelens *et al.*, 1994) are all expressed in the decidua during the peri-implantation period in the mouse (see section below for details of expression pattern). This is relevant to these studies because of supporting evidence for a role of TGF-β molecules, particularly TGF-β1, in the regulation of MMPs and TIMPs in mammalian cells. For example, TGF-β1 added to cell cultures enhances MMPs-2 and -9 transcripts in human and mouse keratinocytes (Salo *et al.*, 1991), promotes MMP-2 mRNA synthesis in human fibroblasts (Overall *et al.*, 1991) and stimulates the transcription TIMPs-1 and -3 messages in $10T_{1/2}$ cells (Leco *et al.*, 1992). TGF-β1 promotes the deposition of ECM by a mechanism that appears to be mediated by the induction of TIMP synthesis, involving the alteration in the local MMP/TIMP balance in tissues. Finally, addition of TGF-β1 to endometrial stromal cells stimulates MMP-9 and TIMPs-1 and -3 steady state mRNA levels (Bany and Schultz, 2000). Thus, TGF-β1 may be an intermediate player in the control of MMP and TIMP

expression and ECM deposition in uterine cells during decidualization.

1.3 TGF-β1 Expression in the Mouse Uterus During the Peri-Implantation Period

By *in situ* hybridization, TGF-β1 mRNA was localized primarily in the luminal and glandular epithelium of the uterus during the pre-implantation period (day 1-4) of pregnancy in the mouse but during the implantation period (days 5-8), it was found to be localized in the decidua. Similar results were obtained by immunohistochemical analysis. Interestingly, at day 5 when implantation events are just initiated, TGF-β1 staining was still observed in the luminal epithelium but intense extracellular staining was observed in the primary decidual zone (PDZ) and the decidualizing stroma surrounding the PDZ (reviewed in Roelen and Mummery, 2000; Tamada *et al.*, 1990). By days 6 and 7, staining becomes detectable in the secondary decidual zone and then in the decidua at the mesometrial pole (day 8).

TGF-β2 molecules exhibit a similar pattern to that of TGF-β1 with expression in the luminal epithelium and glandular epithelium of day 1-4 of the pre-implantation period and expression in the decidua in the implantation/post-implantation period (Das *et al.*, 1992). TGF-β3 is also expressed but not until days 7 to 8 and its expression is confined to the myometrial layer of the uterus (Das *et al.*, 1992) so it does not appear to be localized in the correct site to have a functional role in the implantation process.

The cell surface proteins that can bind TGF-βs with high affinity have been identified and have been designated receptor type I, type II and type III (Massague, 1990). Type I and Type II receptors must form a heteromeric complex to signal and bind TGF-β1 and β3 with high affinity, but TGF-β2 with lower affinity. However, in the presence of Type III receptor, the affinity of the type I and II heteromeric complex for TGF-β2 becomes equal to that of TGF-β1 and β3 (Massague, 1990). While there is differential expression of TGF-β receptor isoforms in embryonic tissues during the peri-implantation period, all TGF-β receptor types are expressed during the implantation period in the uterus and decidua (Roelen *et al.*, 1994). The temporal and spatial pattern of the appropriate receptor for signaling (Tamada *et al.*, 1990; Roelen *et al.*, 1994) has prompted these authors to suggest that TGF-β1 may, indeed, be involved in tissue remodeling, decidualization, and placentation.

1.4 Subtilisin-Like Proprotein Convertases

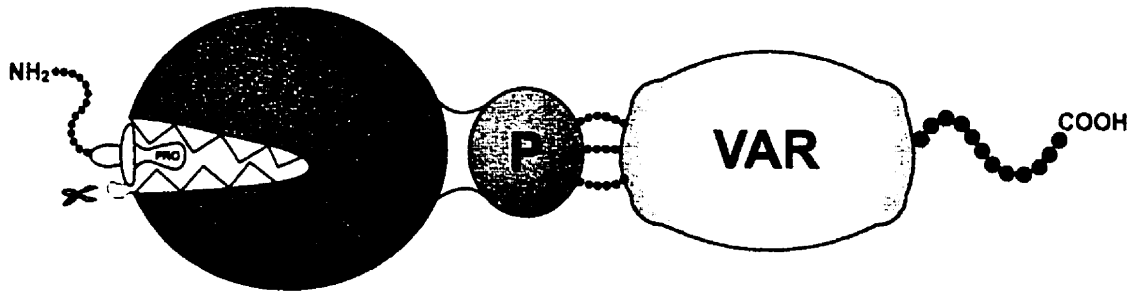
Proprotein convertases were first discovered in 1990 as a family of mammalian secretory processing enzymes that are homologous to the yeast processing protease kex 2, or kexin (reviewed in Seidah and Chretien, 1994; Seidah and Chretien, 1997; Nakayama, 1997; Steiner, 1998; Zhou *et al.*, 1999; and Bergeron *et al.*, 2000). This family of proteolytic enzymes have been called subtilisin-like proprotein convertases (SPC or PC) due to the homology of their catalytic domains to that of the bacterial serine protease subtilisin (Steiner,

1998). Thus far, seven members of the SPC family have been identified and characterized, they are SPC1/SPC3, SPC2, furin/PACE (Paired Amino acid Converting Enzyme), SPC4, PACE4, SPC5/SPC6, and SPC7/LPC/SPC8 (see Seidah and Chretien [1997] regarding the nomenclature used in this report). Alternate splicing of SPC mRNA gives rise to variant isoforms of the SPCs. Proteolytic processing of pro-proteins by the convertases occurs either in the constitutive secretory pathway (in the TGN and the constitutive vesicles derived from the TGN) or the regulated secretory pathway (in the dense core vesicles).

The SPC family of enzymes profoundly affects cellular communication, differentiation, and metabolic activity by determining the cell-type and time at which biologically active products are derived from a given inactive precursor protein (Seidah and Chretien, 1997). The seven members of the SPC family all contain a well conserved signal peptide, an amino-terminal pro-region (*Pro*), a catalytic domain (*CAT*), and a P domain (*P*) (Figure 5; Zhou *et al.*, 1999). Within the catalytic domain of each SPC, there is a conserved catalytic triad of aspartic acid, histidine and serine, of which the catalytically important asparagine is involved in oxyanion stabilization. The P domain may have a regulatory role to influence the more acidic pH optima and marked calcium dependency of some SPCs (Steiner, 1998). In addition, the P domain seems to stabilize the catalytic domain structurally. Variation amongst the various SPC members is contained in the variable C-terminal domain (*VAR*) which includes variations in Cys-rich regions, transmembrane and cytosolic domains, amphipathic helices, and sorting domains (Bergeron *et al.*, 2000).

Figure 5: Structural Features of the Members of the Mammalian Family of Subtilisin-Like Proprotein Convertases

The seven members of the SPC family all have well-conserved signal peptides, pro-regions (*Pro*), catalytic domains (*CAT*), and P domains (*P*). The SPCs differ in their C-terminal domains (*VAR*). Activation of the SPCs results from the autocatalytic cleavage and release of the pro-domain (represented by the scissors). (Modified from Zhou *et al.*, 1999)



SPC3, SPC2, SPC4	Amphipathic Helix
PACE 4, SPC5-A	Cys-rich Domain
Furin, SPC6-B, SPC7	Cys-rich Domain, TM Segment, Cytosolic Tail

Activation of the enzyme is the result of the autocatalytic cleavage and release of the prodomain, which also has two functionally important roles as an intramolecular chaperone and as a competitive inhibitor. For processing of proproteins, SPCs recognize the KR↓ (↓ denotes the site of cleavage) or RR↓ motif but basic residues at the P4 and or P6 position may also contribute to substrate recognition. Furin, the most intensively studied SPC, preferentially recognizes the motif RXK/RR↓ but is also able to cleave RXXR↓ sites in some precursors. Even though each member of this family shares various similarities, the one major difference between each member is the presence of a transmembrane domain. Thus far, only furin, PACE4-E, SPC5-B/SPC6-B, and SPC7 are known to be type-I membrane-bound proteins, cycling to the cell surface via the TGN, thereby processing proproteins within the constitutive secretory pathway (Seidah and Chretien, 1997; Bergeron *et al.*, 2000). In contrast, SPC1, SPC2, various isoforms of SPC4, and SPC5A/SPC6-A, are localized in dense core secretory granules for the processing of precursor proteins within the regulated secretory pathway.

Expression analysis of the various SPCs demonstrates that furin and SPC7 are very widely expressed in all mammalian cells, but furin have not been found to be expressed in the pregnant uterus (Dr. Derrick Rancourt, personal communication). Intracellular localization of the furin and SPC7 proteins is mainly to the TGN, but they are also found in endosomes and at the cell surface (Seidah and Chretien, 1997; Zhou *et al.*, 1999). SPC4 has been found to be exclusively expressed in the germ cells of the testis. The multiple isoforms of

SPC4 have been found in the TGN and/or in dense core secretory granules (Bergeron *et al.*, 2000). SPC1 and SPC2, localizing mostly within the TGN and/or dense core secretory granules, have been found to be expressed primarily in the endocrine and neural cells (Seidah and Chretien, 1997; Zhou *et al.*, 1999). SPC6 expression is detectable early in embryonic development, but exhibits strict temporal and spatial control (Constam *et al.*, 1996; Rancourt and Rancourt, 1997). In adult mice, SPC6 expression is widespread, especially abundant in the intestines, adrenals and the lungs (Lusson *et al.*, 1993; Nakagawa *et al.*, 1993; Seidah and Chretien, 1994). Alternative splicing for SPC6 generates two isoforms, SPC6-A and SPC6-B, leading to its functional diversification. SPC6-A is a soluble form sorted to dense core secretory granules (De Bie *et al.*, 1996; Seidah and Chretien, 1997; Zhou *et al.*, 1999). SPC6-A has been shown to cleave precursors presenting a common RXXR↓XK motif, such as that found in pro-Mullerian Inhibiting Substance within Sertoli cells and in receptor tyrosine phosphatase within endothelial cells. SPC6-B contains a transmembrane domain and a cytosolic tail enabling it to anchor to membranes of the TGN. As it is trafficked to the surface of the cell, it can shed part of its carboxy-terminal segment, resulting in a 170 kDa form which exits from the cell via the constitutive secretory pathway. When furin, SPC6-B, and SPC7 are retained in the TGN, they are provided with strategic access to many precursor proteins that move to the cell surface via constitutive vesicles.

Considering the cleavage characteristics of the SPCs, they are excellent candidate molecules for the processing of a variety of regulated and/or secreted

precursor molecules that contain a SPC cleavage recognition motif. Indeed, it has been shown that SPCs are able to process growth factor precursors, such as TGF-β, bone morphogenetic protein-4 (BMP-4), the insulin-like growth factors (IGF-I and -II), and growth factor receptors such as the insulin, IGF-I, and hepatocyte growth factor receptor, both *in vivo* and *in vitro* (Dubois *et al.*, 1995; Seidah and Chretien, 1997; Constam and Robertson, 1999; Bergeron *et al.*, 2000).

There is accumulating evidence that furin, SPC4, and/or SPC6 proteolytically activate bone morphogenetic proteins (members of the TGF-β family) during embryonic development and regulate axis formation in embryos. (Constam *et al.*, 1996, Rancourt and Rancourt, 1997; Cui *et al.*, 1998; Constam and Robertson, 2000). SPC4 and SPC6 expression during mouse development is highly regulated and prominent only at discrete sites during various stages of development. Correlative studies have found that SPC4 and SPC6 expression in developing limbs overlaps with the expression of BMP-2, -4, and -7. Recently, Cui *et al.* (1998) attempted to provide direct evidence for the proteolytic activation of BMP-4 by furin and/or SPC6 during vertebrate embryonic development by using a bioengineered serpin, α1-antitrypsin Portland (α1-PDX), which is thought to be a potent inhibitor of furin and SPC6-B activities at low levels of the inhibitor (Jean *et al.*, 1998). α1-PDX was engineered by Anderson *et al.* (1993) as a variation of the α1-AT Pittsburgh (α1-AT Pittsburgh), which is a naturally occurring mutant protein of α1-antitrypsin (α1-AT; Benjannet *et al.*, 1997; Tsuji *et*

al., 1999). In humans, α1-AT is the physiological inhibitor of neutrophil elastase and a mutation in the α1-AT reactive site converts it into α1-AT Pittsburgh, changing the serpin's specificity from an inhibitor of elastase to an inhibitor of thrombin. In the Cui *et al.* (1998) studies performed with *Xenopus* embryos, it was found that dorsalization of mesoderm and direct neural induction results from the ectopic expression of α1-PDX. The dorsalization effect phenocopies the effect of blocking endogenous BMP activity. Also, the ventralization of embryos resulting from the overexpression of BMP-4 was rescued by ectopic expression of α1-PDX. Reversal of α1-PDX-mediated patterning defects was achieved by co-expressing a downstream component of the BMP-4 signaling pathway. Furthermore, it was found that α1-PDX entirely blocks the cleavage of BMP-4 in an *in vivo* oocyte translation assay. In their report, Cui *et al.* (1998) provided evidence that α1-PDX can block the activity of the endogenous protease(s) to inhibit BMP activity upstream of receptor binding, to make the claim that either furin and/or SPC6 is responsible for the proteolytic activation of BMP-4, a TGF-β family member. In addition, it has been found that in cell transfection assays, SPC6-A dramatically enhances BMP4 precursor cleavage (Constam and Robertson, 2000). However, the conclusion that furin and/or SPC6 were solely responsible for the processing of BMP4 was later disputed by Tsuji *et al.* (1999). Tsuji *et al.* (1999) found evidence that PACE4 was also able to form a SDS-stable acyl intermediate with α1-PDX, *in vivo* and *in vitro*, suggesting that α1-PDX can also act as an inhibitor of PACE4.

1.5 Research Proposal Overview

In implantation, proteinases mediate embryo-uterine integration through tissue remodeling, activating latent regulatory proteins including cytokines and growth factors, and inducing intracelluar signaling through extracellular protein cleavage. The maintenance of normal implantation is dependent on the proper expression of extracellular matrix (ECM) degrading proteinases. Aberrant expression of these proteinases will result in implantation disorders, but many single gene mutants of proteinases or protease inhibitors exhibit no decline in reproductivity, therefore suggesting very complex relationships amongst the various molecules involved in the process of implantation. Many cytokines involved in implantation are secreted as latent proteins, ie. IGFs and TGF-βs.

The SPC family has been found to activate a number of peptide hormones such as insulin, PDGF, IGFs and TGF-βs. Dr. Derrick Rancourt and his research team had previously screened for subtilisin-like proprotein convertase expression during implantation and isolated a proprotein convertase (SPC6) which may be an important regulator of the uterine environment during implantation and placentation processes. SPC6 is expressed during the implantation period and during embryogenesis. In implantation, it is hypothesized that SPC6 cleaves latent TGF-β to activate it, which in turn regulates the expression of TIMP-3.

Preliminary expression studies of SPC6 revealed a narrow period of expression starting precisely at the day of implantation of the embryo that appears to coincide with the temporal and spatial pattern of TIMP-3 expression (Rancourt, unpublished data). In this work, the mouse has been used as the

animal model for implantation studies because human and mice both exhibit the most invasive type of embryo implantation. The long term aim of these studies is to further our understanding of the molecular basis of mammalian embryo implantation and also the diseases of implantation.

To extend previous preliminary work on SPC6 expression in the mouse (Rancourt and Rancourt, 1997), this thesis reports on experiments that were conducted using Northern blot analysis and reverse-transcription polymerase chain reaction (RT-PCR) methods to examine the tissue distribution of SPC transcripts in the adult mouse, the temporal pattern of expression of the SPC6 gene in several mouse tissues during embryonic development and the expression of SPC6 gene in the uterus during the peri-implantation period. Experiments were also carried out on artificial, oil-induced deciduomas to evaluate SPC6 expression in uterine endometrial stromal cells independent of embryonic contributions to the implantation site. *In situ* hybridization was used to show that SPC6 is strongly expressed in endometrial stromal cells during this artificial decidualization reaction.

To test the hypothesis that SPC6 may play a role in the uterus through activation of TGF-β family members, RT-PCR was used to verify expression of TGF-β1 and TGF-β2 genes in the implantation site and attempts were made to resolve latent and processed forms of TGF-β1 from decidual tissue on Western blots. Unfortunately, this technique proved to be too insensitive to detect the levels of TGF-β1 present in the decidua. Thus, an alternative, indirect approach

was developed to test whether SPC6 might act on TGF-β1 in the uterus. An inhibitor of SPC6 was added to cultures of endometrial stromal cells in which it has previously been shown that TGF-β1 induces increased levels of TIMP-3 mRNA expression. However, even at very high concentrations of the SPC6 inhibitor, no influence on the levels of TIMP-3 was detectable. These findings are discussed in relation to the possible role that SPC6 plays in the implantation process.

2. Materials And Methods

2.1 Animals and Tissue Collection

All procedures involving animals were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Calgary Animal Care Committee. CD1 mice (6-8 weeks old, 22-25 g) were obtained from Charles River Breeding Laboratories (Lachine, Canada) and were housed under temperature- and light-controlled conditions (07:00 to 19:00 lights on) with free access to food and water.

To obtain pregnant females, female mice were placed with fertile males in a ratio of one-to-one. The presence of a vaginal mucus plug the next morning was indicative of copulation and fertilization during the previous dark cycle. Detection of the vaginal plug was used to designate day 0.5 of pregnancy. Embryos were dissected from the decidua and uterus at various stages of gestation, according to the need of the experiment. Embryonic tissues were obtained under a dissection microscope after washing with ice-cold Phosphate Buffered Saline (PBS: composed of 0.137 M NaCl, 2.88 mM KCl, 6.48 mM Na_2HPO_4 anhydride, and 1.47 mM KH_2PO_4).

2.2 Artificial Decidualization of Mouse Uterus

The uteri of mice were induced to undergo the decidualization reaction artificially, in vivo, similar to the procedures that are used to artificially decidualize rat uteri (Yee and Kennedy, 1988; Kennedy and Ross, 1997). The mice were ovarectomized under methoxyflurane (Metafane) anesthesia (M.T.C.

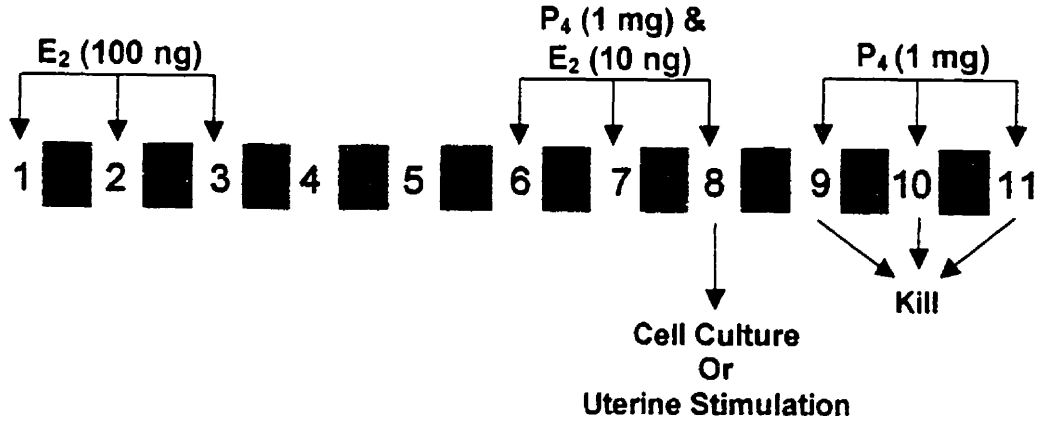
Pharmaceuticals, Cambridge, Canada) and allowed to rest for 4-5 days prior to being sensitized for artificial decidualization with a hormonal regimen (see Figure 6 for schematic representation of hormonal sensitization protocol). Estradiol 17-β and/or progesterone, in 0.1 ml sesame oil (Sigma) was then administered subcutaneously at 09:00 h over 8 days, as described in Milligan and Mirembe (1985). Briefly, the animals received 100 ng of estradiol on days 1 to 3, then 1 mg of progesterone plus 10 ng of estradiol on days 6 to 8. On the morning of day 8, when the uteri are optimally sensitized for a deciduogenic stimulus, the mice were used for oil-induced decidualization or the uteri were dissected for endometrial stromal cell isolation (see below). In order to obtain oil-induced decidualization, 15 μl of sesame oil was injected into the lumen of one uterine horn (stimulated horn) between 11:00 h and 13:00 h on Day 8. The other uterine horn (non-stimulated horn) was not injected with oil and served as a control. On days 9 onwards, the mice were injected with 1 mg of progesterone (subcutaneously) to maintain the decidual response. At various hours after the unilateral intra-luminal oil injection, the mice were sacrificed by cervical dislocation and the uterine horns were removed by dissection.

2.3 Endometrial Stromal Cell Cultures

To obtain optimally sensitized uteri for decidualization, ovarectomization and hormone treatment was identical to that used for induction of oil-induced deciduomas (Figure 6). On Day 8 of hormone treatment, the mice were sacrificed and their uterine horns split longitudinally and incubated with

Figure 6: Treatment Protocol for Uteri Sensitization

Schematic representation of the treatment protocol administered to ovarectomized mice to obtain differentially sensitized uteri for decidual cell cultures or oil-induced artificial decidualization *in vivo*. The dark blue squares indicate dark cycles and light blue squares indicate light cycles within a 24 hour period. E_2 = Estradiol, P_4 = Progesterone. (Modified from Kennedy and Ross' [1997] protocol for rat uteri sensitization)


E_2 (100 ng)
P_4 (1 mg) &
E_2 (10 ng)
P_4 (1 mg)
1
2
3
4
5
6
7
8
9
10
11
Cell Culture
Or
Uterine Stimulation
Kill

pancreatin and dispase II (Roche Diagnostics) for the liberation of epithelial cells. The uterine horns were then incubated with collagenase (Sigma) to release the endometrial stromal cells. After collagenase treatment, the stromal cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM):F12 (Gibco-BRL) nutrient mixture containing 10% heat-inactivated charcoal-stripped fetal calf serum (v/v, Gibco-BRL), Antibiotic-Antimycotic (100X: 10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B in 0.85% saline; Gibco-BRL) and filtered through nylon mesh (70 μm) to remove contaminating glands. The cells were counted in a hemocytometer and plated at a density of $5x10^5$ cells in 0.5 ml of medium. After the stromal cells had undergone a differential attachment period of 2 hours at 37°C under 5% CO_2:95% air, the old medium was removed and replaced with fresh medium containing 10% serum. This point was designated time 0 hour of Day 1 of culture.

The cells were either treated with the SPC inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-CH_2Cl; Bachem), immediately after the attachment period or were cultured with 10% serum for the first 24 hours, then treated with the inhibitor on Day 2. Dec-RVKR-CH_2Cl was dissolved in water and mixed into the culture media (with 10% serum) in various concentrations. RNA was extracted from the cells at designated hours after addition of inhibitor.

2.4 RNA Extraction

Total RNA was initially obtained from tissues and embryos using the guanidine-isothiocyanate procedure described by Chomczynski and Sacchi, 1987. Later, preference was given to using Trizol® (Gibco-BRL) for more efficient RNA extraction. Dissected tissues and embryos were washed in ice-cold PBS before the process of RNA extraction. For Northern hybridization experiments, the RNA was resuspended in formamide (deionized, OmniPur), quantified and stored at –20°C. For RT-PCR experiments, the RNA was resuspended in distilled water treated with the ribonuclease inhibitor, diethylpyrocarbonate (DEPC-dH_2O) and stored at –20°C.

2.5 Northern Blot Hybridization

2.5.1 Transfer of RNA to Solid Support

Total RNA was resolved on denaturing 1% formaldehyde-agarose gels prepared by dissolving 1 g of agarose (Gibco-BRL) in 73 ml of boiling dH_2O. After the gel had cooled but not yet solidified, 10 ml of 10X MOPS (10X MOPS: 0.4 M MOPS [Sigma], pH 7.0; 0.1 M sodium acetate; 10 mM EDTA) buffer, and 18 ml of formaldehyde (supplied as 37% or 12.3 M solution in H_20; BDH) was added. The gel was mixed and poured in a horizontal gel apparatus.

The RNA samples for loading were first quantified and diluted to 1-2 μg/μl in formamide. Samples for loading contained 10 μl of RNA (1-2 μg/μl) in formamide, 2 μl of 10X MOPS, 4.5 μl of H_2O, and 3.5 μl of formaldehyde. The

samples were then heated to 65°C for 15 minutes to eliminate secondary structures in the RNA and cooled on ice before loading. Prior to loading, 0.5 μl of ethidium bromide (1 mg/ml) was added to the samples for visualization purposes. The denaturing gel was electrophoresed in 1X MOPS as the running buffer. After electrophoresis, RNA was transferred and immobilized onto Hybond NX membrane by capillary transfer with 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). After the transfer, membranes were cross-linked either by baking at 80°C for 1 hour or in an UV-Crosslinker on the "Auto-Crosslink" setting (Stratagene).

2.5.2 DNA Probe Synthesis

The DNA fragments used as probes in these studies were either a 2.8 kb fragment of SPC6 cDNA cloned into the *EcoR*I sites of the pBS KS$^+$ vector (kindly provided by Derrick Rancourt) and a 750 bp fragment of the TIMP-3 gene subcloned into pBS from the TIMP-3 full length cDNA (Leco *et al.*, 1994; the clones were kindly provided as gifts from D. Rancourt and B. Bany, respectively). The probe for hybridization was prepared by restriction digestion of the plasmid with *EcoR*I to release the SPC6 cDNA fragment and with *Hind*III and *BamH*I to liberate the TIMP-3 cDNA fragment. The digestion reaction was condensed (by DNA precipitation or lyophilisation) and resolved on a 1.5% TBE-agarose gel. The appropriate sized fragment was excised out of the gel and the DNA was recovered using the glassmilk technique. The DNA fragment (50-100 ng) was denatured by boiling and cooling, and mixed with 6 μl of a dNTP mixture (Gibco-BRL), 5 μl of 10X Random Primer Buffer Mix (Gibco-BRL), 5 μl α-^{32}P-dCTP (10

μ*Ci*/μl, 3000 *Ci*/mmol, Amersham), 1 μl Klenow fragment (2-3 units, Gibco-BRL), and the total reaction volume was brought up to 50 μl with dH_2O. The reaction was incubated at room temperature, behind plexiglass, for 1 hour. Purification of the probe was accomplished with Nick columns (Sephadex™ G-50, DNA-grade; Amersham Pharmacia Biotech). The specific activity of the probe varied from 3-$5x10^6$ cpm/μg.

2.5.3 Hybridization and Detection

Before the hybridization step, the membrane containing the immobilized RNA was incubated with the pre-hybridization mixture at 65°C for 3-4 hours. The pre-hybridization buffer contained 0.1% sodium pyrophosphate (w/v), 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% SDS, 5X Denhardt's (50X Denhardt's: 1% each of Ficoll [type 400, Sigma], polyvinylpyrrolidone [PVP-360, Sigma], and bovine serum albumin [fraction V, Sigma]), and 100 μg/ml sheared, denatured salmon sperm DNA. After prehybridization, the purified SPC6 probe was added to the prehybridization mix at $1x10^6$ cpm/ml and the hybridization reaction was allowed to proceed overnight at 65°C with gentle agitation.

The next day, the membranes were washed twice in 2X SSC and 0.1% SDS for 15 minutes each and twice in 0.2X SSC and 0.1% SDS for 15 minutes each. All washes were done at 65°C. The membranes were placed against Kodak XOMAT or BMS film (Eastman Kodak Co.) and placed in the –72°C freezer to expose for 48 to 72 hours.

2.6 Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

2.6.1 Reverse-Transcription

Reverse transcription (RT) and multi-plex polymerase chain reactions (PCR) were performed using two sets of primers within one reaction. For the RT reaction, 1 μl of RNA (1 μg/μl), 2 μl of random hexamers (1 μg/μl) and 8 μl of DEPC-dH_2O were initially mixed and incubated for 10 minutes at 70°C. The above mixture was then combined with 4 μl of 5X Reverse Transcription buffer (Gibco-BRL), 2 μl 0.1 M DTT (Gibco-BRL), 1 μl 10 mM dNTPs (10 mM dATP, 10 mM dTTP, 10 mM dGTP and 10 mM dCTP), 1 μl RNAguard (Pharmacia), and 1 μl Superscript II (Gibco-BRL). The reaction was incubated at 42°C for 1 hour. For convenience, master mixes were used to combine common components of the reverse transcription reaction when large batches of reactions were done.

2.6.2 Polymerase Chain Reaction (PCR)

PCR was carried out using two sets of primers, one being either SPC6 or TIMP-3 primers and the other being GAPDH primers to serve as an internal control. SPC6 primers were designed by B. Bany with the Primer Designer computer program and the available SPC-5/6 sequence in Genebank and Database Japan (gb:L14932 and dbj:D12619). The upstream primer sequence for SPC6 was (5')-GCCACTACCATGCTGACAAGAA-(3'), T_m: 73°C. The downstream primer sequence was (5')-TTGAAGAACTGTCCATCCTCG-(3'), T_m: 70°C. PCR with SPC6 primers yielded a 303 bp product. The mouse TIMP-3 primer sequences that were used were taken from Wong *et al.* (1999). The

mTIMP-3 primer sequences used were as follows: (5')-CTTGTCGTGCTCCTGAGCTG-(3') and (5')-CAGAGGCTTCCGTGTGAATG-(3'). PCR with mTIMP-3 primers yielded a 244 bp product. GAPDH primers were used in the same PCR reaction as SPC6 or mTIMP-3 and served as an internal control. The GAPDH primers used were designed by G. Schultz and A. Hogan from published cDNA sequences of the GAPDH gene and were as follows: (5')-ACCACAGTCCATGCCATCAC-(3') and (5')-TCCACCACCCTGTTGCTGTA-(3'). The GAPDH primers amplified a region 450 bp in length within the ubiquitously expressed GAPDH gene.

RT-PCR for TGF-β1 and TGF-β2 transcripts was carried out as described above in the absence of GAPDH primers. Primer sequences are described in Watson *et al.* (1992). For TGF-β1, the upstream primer has the sequence (5')-AAGTGGATCCACGAGCCCAA-(3') and the downstream primer has the sequence (5')-GCTGCACTTGCAGGAGCGCA-(3'). For TGF-β2, the upstream primer has the sequence (5')-AGAAATGTGCAGGATAATTGCTGC-(3') and the downstream primer has the sequence (5')-TTCGATCTTGGGCGTATTTCCAAT-(3'). The expected product sizes for TGF-β1 and TGF-β2 transcripts are 245 bp and 272 bp, respectively.

A standard protocol for PCR was used in which each reaction mixture contained 1X PCR buffer minus magnesium (10X PCR buffer: 200 mM Tris-HCl [pH 8.4], 500 mM KCl; Gibco-BRL), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 2 μl of cDNA template for a 50 μl reaction and 0.025 units/μl

of Taq polymerase (all reagents supplied from Gibco-BRL). The reaction was placed in the thermocycler (GeneAmp PCR System 2400, Perkin-Elmer) at 94°C with the following temperature cycling profile:

i) 94°C for 5 minutes

ii) cycling from 94°C to 55°C to 72°C for 30 seconds on each temperature for 35 cycles

iii) soak at 4°C

PCR products were stored at –20°C until gel electrophoresis analysis could be done. Like the procedure for reverse transcription reactions, common components were combined in master mixes for purposes of setting up large numbers of reactions.

2.6.3 Analysis of PCR Product

Following PCR amplification, 25 μl of the reaction mixture was resolved on a 2% agarose gel (w/v) made with 1X TAE (50X TAE: 242 g Tris-base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA, pH 8.0; dH_2O to 1.0 litre; pH 8.0) containing 0.005% ethidium bromide (10 mg/ml). Products were electrophoresed in 1X TAE buffer along with molecular weight markers (100 bp ladder, Gibco-BRL). After electrophoresis, the gels were photographed under UV illumination.

2.7 *In situ* Hybridization

2.7.1 Tissue Fixation and Embedding

Uteri were harvested from animals artificially induced (with sesame oil) to undergo decidualization from 11:00 h to 13:00 h on appropriate days. The uteri were promptly washed in RNAse-free 1X PBS (0.1% DEPC-treated 10X PBS) and fixed in cold 4% paraformaldehyde (Sigma)/1X PBS (pH 7.4) overnight at 4°C with gentle agitation. After fixing, the tissues were washed and dehydrated through a series of graded ethanol washes. Washes were one hour each through increasing concentrations of ethanol: 70%, 90%, 95%, 100%. Then, tissues were washed with ethanol/xylene (1:1) once followed by two changes of xylene for one hour each.

The tissues were transferred from xylene to 1:1 xylene/paraffin wax (Paraplast X-tra, Oxford Labware) and incubated at 60°C for one hour. This step was followed by two changes of paraffin wax with the last change incubated at 60°C overnight under vacuum conditions. The next day, the uterine tissues were placed into a mold filled with wax in an appropriate orientation to allow for cross-sectioning. Each paraffin wax block contained a segment of stimulated horn and non-stimulated horn.

To obtain 10 μm sections, a Reichert-Jung microtome (1130/Biocut) was used (courtesy of the Rancourt lab, University of Calgary). Sections were place on glass microscope slides (Micro Slides, Superfrost ® Plus, VWR Scientific) and allowed to dry overnight at 42°C on a slide warmer. The sections were then stored in a clean, dry slide box.

2.7.2 Riboprobe Synthesis

The SPC6 fragment (~380 bp in length) used for the synthesis of the probe represents the 5'-end of the SPC6 cDNA. The DNA fragment was cloned in a pBS-KS$^+$ vector (cloned by S. Rancourt) and excised by restriction digestion using *EcoR*I and *BamH*I. For anti-sense probe transcription, 3 μg of the plasmid was linearized with *Xba*I (Gibco-BRL) while 3 μg of the plasmid was linearized with *Hind*III (Gibco-BRL) for sense probe transcription. After linearization, 0.5 μg of the DNA was resolved on a 1% agarose gel to check for complete digestion. If complete digestion was achieved, the DNA was treated with proteinase-K (Roche Diagnostics) at 10 μg/ml for 30 minutes at 37°C. RNAse-free conditions were maintained from this point forward. After proteinase-K treatment, the mixture was extracted with phenol/chloroform and precipitated using 0.3 M NaCl (RNAse-free) and 2 volumes of 100% ethanol at –20°C for a minimum of 30 minutes. For transcription of the digoxygenin (DIG)-labelled riboprobe, the following components were place directly into the microcentrifuge tubes containing the dried, linearized DNA pellet (all reagents for this reaction were supplied by Gibco-BRL): 10 μl 5X Transcription buffer; 5.25 μl of 0.1 M DTT; 10 μl 2.5 mM NTPs (2.5 mM DIG-11 UTP mix: 1 μl of each 100 mM CTP, GTP, ATP; 6.5 μl 10 mM UTP; 3.5 μl DIG-11 UTP; 27 μl DEPC-dH_2O to give final volume of 40 μl); 50-100 units of enzyme (T3 or T7 depending on anti-sense or sense reaction); and DEPC-dH_2O to a final volume of 50 μl. The reaction was incubated at 37°C for 2 hours. After transcription, 1 μl of 1 mg/ml RNAse-free DNAse (Roche

Diagnostics) was added and incubated at 37°C for another 15 minutes to digest the DNA template. The DIG-labeled riboprobe was precipitated in 0.3 M NaCl and 2.5 volumes of ethanol, placed at –20°C for at least 1 hour or overnight and spun down in a microcentrifuge. After precipitation, the RNA pellet was resuspended in 50 µl of DEPC-dH_2O and stored at –20°C until ready to use.

2.7.3 Quantification of Probe and Checking of DIG Incorporation

Probe concentration was estimated by electrophoresis in a RNAse-free, 1.2% agarose-formaldehyde denaturing gel by comparing intensity of ethidium bromide staining to known amounts of DNA standards run in parallel slots in the gel. DIG incorporation into the probe was measured using a spot test. One µl of the probe was spotted onto a Hybond N^+ filter (Amersham Pharmacia Biotech) in serial dilutions in the range of 10^{-2} to 10^{-5}. Also included on the filter was a spot of undiluted probe (1 µl) and a negative control using 1 µl of non-specific DNA.

After immobilization of the RNA onto the filter via UV-Crosslinking (Stratagene), the filter was blocked at room temperature for 30 minutes using 1% Blocking Reagent (Roche Diagnostics) or 5% skim milk powder (Carnation) in 1X spot buffer (1X spot buffer: 100 mM Tris-HCl, pH 7.5; 150 mM NaCl). The filter was then washed twice for 15 minutes each in 1X spot buffer and incubated with alkaline phosphatase conjugated anti-DIG antibodies (Roche Diagnostics) at 1:5000 dilution in 1X spot buffer for 30 minutes at room temperature. The filter was washed again twice in 1X spot buffer for 15 minutes each after the incubation step. The color reaction was developed with 4.5 µl/ml nitro-blue

tetrazolium salt (NBT: 75 mg/ml in 70% DMF; Roche Diagnostics) and 3.5 µl/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP: 50 mg/ml in 100% DMF; Roche Diagnostics) in 1X alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 50 mM $MgCl_2$; 100 mM NaCl). From experience, good incorporation of DIG in the riboprobes results in color development at a dilution of 10^{-4} in 30 to 60 minutes. Only probes containing this level of DIG incorporation were used. The color reaction was stopped by washing twice for 5 minutes with 5X TE (10X TE: 100 mM Tris-HCl, pH 7.6; 10 mM EDTA, pH 8.0).

2.7.4 Hybridization

Throughout the hybridization procedure, it was important to maintain RNAse-free conditions. The slides with sections of tissue embedded in paraffin wax were first de-waxed and rehydrated for the times indicated by immersing in the following solutions contained in RNAse-free Conklin jars or slide holders:

i) 2 minutes in xylene (twice)

ii) 2 minutes in 100% ethanol

iii) 2 minutes in 80% ethanol

iv) 2 minutes in 50% ethanol

v) 5 minutes in 2X SSC

The sections were incubated for 5 minutes at room temperature with 20 µg/ml freshly prepared proteinase-K in proteinase-K solution (20 mM Tris-HCl, pH 7.5; 5 mM EDTA). The slides were acetylated with a freshly prepared mixture of 0.1 M triethanolamine (BDH) and 0.56% (v/v) acetic anhydride (Analar) solution for 10 minutes at room temperature. After acetylation treatment, the

slides were washed twice for 5 minutes in 2X SSC and then incubated with prehybridization solution (50% formamide [deionized, Gibco-BRL]; 5X SSC, pH 5; 1% SDS; 50 mg/ml heparin [Sigma]; 50mg/ml Torula RNA [phenol/chloroform extracted several times, precipitated and resuspended in DEPC-dH_2O and stored in –20°C until ready to use; Roche Diagnostics]). The prehybridization solution was made in 50 ml stocks and stored at –20°C. Before prehybridization, the slides were divided into sections with an immunological staining PAP pen (Daido Sangyo Co., Ltd. Japan; distributed by Accurate Chemical & Scientific Corp., Westbury, N. Y.). The slides were laid flat on a petri dish inside a humidified chamber (Tupperware box with tight lids; petri dish containing the slide was put on a layer of paper towels moistened with 50% formamide and 2X SSC or dH_2O). Prehybrization solution was added onto the slides sparingly (approximately 50 μl per slide in total). The chamber was closed tightly to prevent evaporation and subsequent drying of slides, and was then incubated at 60°C for at least 2 hours before the addition of the DIG-labeled riboprobe. After prehybridization, approximately 200-500 ng/ml of the riboprobe was added to fresh prehybridization buffer and hybridization was allowed to proceed overnight at 60°C.

Washes – RNAse-free conditions were not required from this step forward, and 50 ml of each solution (pre-warmed) was used for each washing step. A Pasteur pipette was used to gently rinse the slides 3 times with the first wash solution. The washing steps were as follows:

i) 2 x 30 minutes in 50% formamide, 2X SSC at 60°C

ii) 3 x 5 minutes in 2X SSC at 37°C

iii) 1 x 30 minutes in 2X SSC with 20 μg/ml RNAse A (Roche Diagnostics) at 37°C

iv) 2 x 30 minutes in 50% formamide; 2X SSC at 60°C

v) 3 x 5 minutes in Tris-buffered saline with Tween-20 or TBST (1X TBS: 25 mM Tris-HCl, pH 7.6; 140 mM NaCl; 2.7 mM KCl, 0.1% Tween-20 [Sigma]) at room temperature.

DIG-probe Detection – After the final TBST wash, the slides were pre-blocked by covering with TBST containing 10% heat inactivated sheep serum (Sigma) to reduce non-specific binding of alkaline phosphatase conjugated anti-DIG antibodies. The slides were again placed in the humidified chamber and incubated at room temperature for at least 2 hours. After the slides were blocked, alkaline phosphatase conjugated anti-DIG antibodies were added to fresh TBST/10% heat inactivated sheep serum diluted by a factor of 1:2000 to 1:5000. The antibody was incubated with the slides overnight at 4°C. After incubation with antibody, the slides were rinsed several times with TBST and then 3 times for 5 minutes with TBST (50 ml or more per wash). The slides were then washed 3 times for 30 minutes each in TBST with gentle rocking (50 ml or more per wash). To prepare the slides for the color reaction, they were washed twice for 5 minutes each in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 50 mM $MgCl_2$; 100 mM NaCl; 0.1% Tween-20; 0.5 mg/ml levamisole [Sigma]). The substrates are added to the alkaline phosphatase buffer at 4.5 μl/ml NBT and 3.5 μl/ml BCIP. Color development was allowed to proceed in the

dark until satisfaction with the signal was achieved (one hour to overnight). When signal intensity was optimal, the color reaction was stopped using PBST (1X PBS with 0.1% Tween-20).

2.7.5 Fixing and Mounting of Slides

The slides were fixed, dehydrated, and counter-stained by passage for the times indicated through the following series:

i) 2 minutes in 50% ethanol

ii) 2 minutes in 80% ethanol

iii) 2 minutes in 95% ethanol

iv) 2 minutes in 0.01% eosin/95% ethanol

v) 2 minutes in 95% ethanol

vi) 2 minutes in 100% ethanol

vii) 2 minutes in xylene – twice

For mounting of slides, Permount (Fisher Scientific) was spread over the slides and the slides were covered with coverslips. The slides are allowed to dry for at least two days underneath a weighted piece of glass (~1 kg). The slides were cleaned of excess Permount with xylene before being examined under a dissecting microscope.

2.8 Western Blots

2.8.1 Preparation of Sample

Pregnant, female mice were sacrificed by cervical dislocation at days 5.5, 6.5 and 7.5 of pregnancy and their uteri were obtained by dissection. After separating the uterus into implantation (IS) and non-implantation (NIS) sites, 100 mg of tissue was placed in a pestle-fitted microcentrifuge tube (VWR) containing 1 ml of protein extraction buffer (20 mM Hepes, pH 7.8; 450 mM NaCl; 0.2 mM EDTA; 0.5 mM DTT; Complete, Mini, EDTA-free, protease inhibitor cocktail tablet [one tablet dissolved in 10 ml of protein extraction buffer, Boehringer Mannheim]). The tissue was homogenized and the proteins were further released from the cells by freeze-thawing three times by transfer between dry ice/ethanol and a 37°C water bath. Cellular debris and other solid material were pelleted by centrifugation and the supernatant was transferred to a fresh tube for storage at –70°C.

2.8.2 SDS-PAGE Gel Electrophoresis

For western blot analysis, SDS-PAGE was used to resolve the polypeptides. To resolve a small protein, such as the processed form of TGF-β1 (12.5 kDa), a 15% separating gel was made beneath a 4% stacking gel according to the protocol described in Sambrook *et al.* (1989). Casting of the gel was performed with a Bio-Rad vertical gel apparatus. Before loading the samples into the lanes, the protein extract was mixed in a 1:1 ratio with 2X protein gel loading buffer (for 100 ml loading buffer: 25 ml 4X Tris-HCl/SDS pH 6.8 [0.5 M Tris-HCl, pH 6.8; 0.4% SDS]; 20 ml glycerol; 4 g SDS; 2 ml β-

mercaptoethanol; 1 mg bromophenol blue; dH_2O to 100 ml). The samples were boiled for 2-3 minutes and cooled on ice. The first lane of the gel was loaded with Rainbow Markers (Amersham Pharmacia Biotech) and 20-40 μg of protein was loaded in each of the following lanes. The gel was subjected to electrophoresis at 150V for one hour in Tris-glycine electrophoresis buffer (25 mM Tris-base, 250 mM glycine [electrophoresis-grade], 0.1% SDS [electrophoresis grade]; Sambrook *et al.*, 1989). After the gel was finished running, it was either stained with Coomassie Brilliant Blue to compare band intensity for equal loading or electro-transferred to Hybond-P (Amersham Pharmacia Biotech) for western blotting.

2.8.3 Coomassie Brilliant Blue Staining

After electrophoresis, the SDS-PAGE gel was stained according to the procedure outlined in Sambrook *et al.* (1989) to demonstrate the amount of protein loaded in each lane. In brief, the gel was separated from the gel apparatus and placed in at least 5 volumes of staining solution (0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:dH_2O [1:1 v/v] and 10 ml of glacial acetic acid) on a rotating platform for a minimum of 4 hours at room temperature or overnight at 4°C. After staining, the gel was destained using the rapid destain method by soaking the gel in 30% methanol, 10% acetic acid at room temperature for 2-4 hours, on a rotating platform. The destaining solution was changed about once every 1.5 hours. After the destaining process, the gel was rinsed clean of methanol and acetic acid and stored in bags in dH_2O or dried.

2.8.4 Transfer of Protein to Solid Support

If the gel was to be transferred, it was removed from the gel apparatus and the stacking gel excised out of the SDS-PAGE gel. To prepare 1 L of the transfer buffer, 3.03 g of Tris-base, 14.4 g of glycine, 200 ml methanol, and dH_2O to 1 L were mixed together and the solution cooled to 4°C. Before the transfer, the porous pads, the Whatman papers, and the gel were equilibrated in the transfer buffer. The Hybond-P membrane was first wet with methanol and rinsed with dH_20 before equilibration in the transfer buffer. To prevent trapping air bubbles in the transfer apparatus, the next step was submerged in transfer buffer. The Bio-Rad transfer unit was used for all transfers and the "gel transfer sandwich" was set up in the following manner in the transfer apparatus:

i) anode

ii) porous pad

iii) Whatman paper x 2 pieces

iv) Hybond-P membrane

v) gel

vi) Whatman paper x 2 pieces

vii) porous pad

viii) cathode

The transfer apparatus was then placed inside the running tank with cold transfer buffer and connected in the correct orientation so that the protein from the gel migrated in the direction of the Hybond-P membrane. The transfer was done at 100V for 1 hour. After the transfer was completed, the membrane was wet in

methanol twice and allowed to dry to immobilize the protein onto the membrane.

2.8.5 Immunological Detection of Protein

After immobilization of proteins onto Hybond-P, the membrane was blocked in 5% skim milk powder in 1X TBST (w/v; Carnation) for at least 1 hour at room temperature or overnight at 4°C on a rotating platform. Anti-TGF-β1 (200 μg IgG in 1 ml of PBS containing 0.1% sodium azide and 0.2% gelatin; Santa Cruz Biotechnology, Inc.) was used as the primary antibody. This antibody was an affinity-purified rabbit polyclonal antibody raised against a well-conserved amino acid sequence corresponding to the carboxy terminus of the precursor form of human TGF-β1. Anti-TGF-β1 reacts with TGF-β1 of mice, rats, and humans and it is able to recognize both the precursor and active forms of the TGF-β1 protein, but it is not cross-reactive with other TGF-β family members. The primary antibody was added to fresh blocking buffer in 1:2000 to 1:5000 dilution and incubated for at least 1 hour at room temperature or overnight at 4°C with gentle agitation. After incubation with anti-TGF-β1, the membrane was washed three times in 1X TBST for 10 minutes each wash (approximately 50 ml per wash) on a rotating platform. After washing, the membrane was incubated with HRP-conjugated anti-rabbit antibody (Santa-Cruz Biotechnology, Inc.) in 1:5000 dilution in blocking buffer for 1 hour at room temperature. The washes were repeated for the secondary antibody (3 times with TBST for 10 minutes each wash at room temperature). After the final wash, excess TBST was drained from the membrane and ECL (Amersham Pharmacia Biotech) was applied

directly to the membranes evenly, for chemiluminescence detection. A few minutes after ECL application, excess fluid was drained and the membrane was wrapped in plastic and exposed to Kodak BML film (Eastman Kodak Co.). Exposure time varied from 2 seconds to 30 minutes, depending on the intensity of the signal.

3. Results

3.1 Characterization of SPC6 Expression in Adult Mouse Tissues

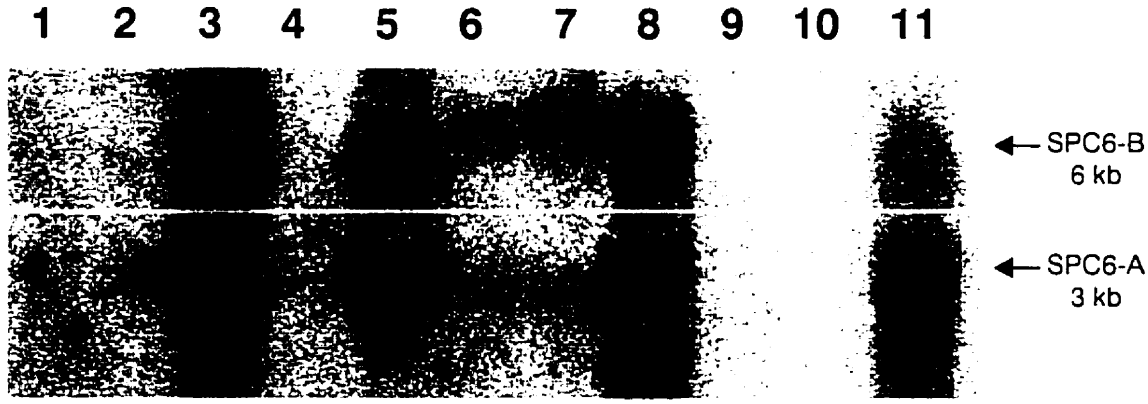
To determine which tissues express SPC6 most abundantly, RNA was extracted from the brain, heart, kidneys/adrenals, liver, large intestines, lungs, skeletal muscles, small intestines, spleen, stomach, and the uterus of adult mice and was resolved electrophoretically on a 1% formaldehyde agarose gel. The RNA was transferred to Hybond NX membrane and a 2.8 kb insert of pSPC6, radioactively labeled with ^{32}P by random priming, was used as probe. After hybridization, two bands of approximately 3 kb and 6 kb in size, representing the transcripts of the SPC6-A and SPC6-B isoforms respectively, were detected by autoradiography.

In adult mice, it was found that SPC6 is most abundantly expressed in the kidneys/adrenals, intestines (small and large), skeletal muscle, and uterus (Figure 7). SPC6 expression could also be detected, to a lesser degree, in RNA extracted from lungs (Figure 7). In the kidneys/adrenals, large intestines, lungs, and skeletal muscles, the SPC6-B transcript was more abundant than the SPC6-A transcript (Figure 7). In the small intestines and the uterus, the smaller SPC6-A transcript was found to be more highly expressed than SPC6-B. The differential expression of SPC6-A and SPC6-B transcripts suggest that in the tissues which express SPC6, distinct preference is given to expression of one of the isoforms in different tissue types. The absence of signal was not due to unequal loading of samples since 28S and 18S rRNA bands were detectable by ethidium bromide staining in all lanes. This experiment was carried out on three

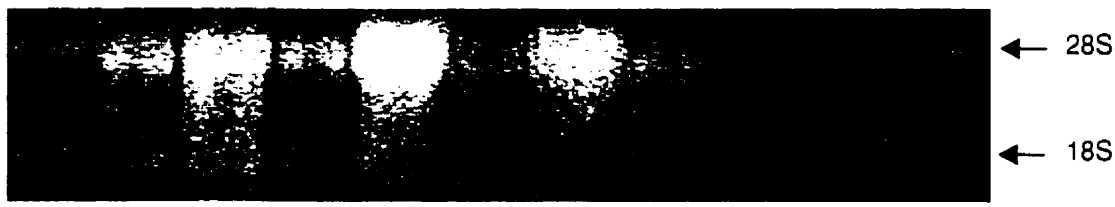
Figure 7: Analysis of SPC6 Gene Expression in Adult Mouse Tissues

A) Northern blot analysis was carried out on aliquots (~20 μg) of total RNA extracted from various adult tissues, blotted onto Hybond NX membrane, and hybridized with a ^{32}P-labeled SPC6 probe. Autoradiographic exposure time was 3 days at -70°C. Lanes: 1 - brain tissue, 2 - heart, 3 - kidneys/adrenals, 4 - liver, 5 - large intestines, 6 - lung, 7 - skeletal muscles, 8 - small intestines, 9 - spleen, 10 - stomach, 11 - uterus. B) Ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane.

A.



B.



independent sets of RNA preparations and identical results were obtained in each case.

3.2 Characterization of SPC6 Expression in Embryonic Mouse Tissues

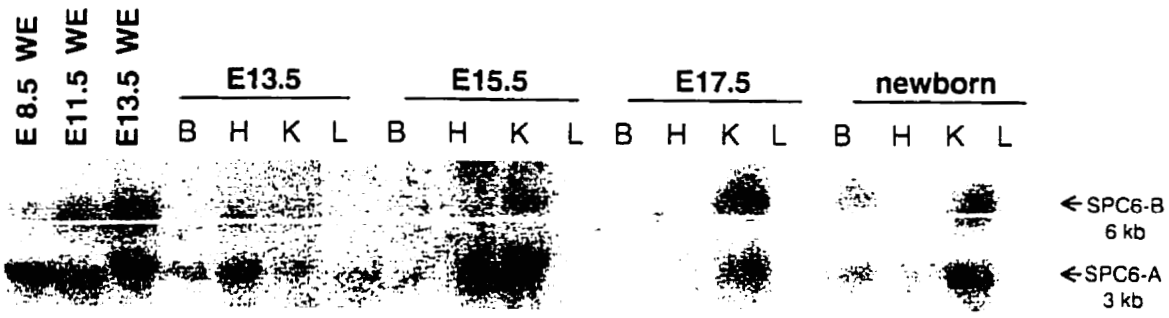
To determine if SPC6 expression was developmentally regulated, RNA was obtained from whole embryos dissected free of extra-embryonic material at days 8.5, 11.5, and 13.5 of gestation. The brains, hearts, kidneys/adrenals and livers of embryos at gestation days 13.5, 15.5, and 17.5/18.5, and newborn mice were also dissected and prepared for RNA extraction to ascertain whether these organs expressed SPC6 mRNA during embryonic development. The results of Northern blots of these samples are shown in Figure 8.

In whole embryos, the SPC6-A transcript was detectable in higher abundance than the SPC6-B transcript until day 13.5 of gestation (Figure 8). In dissected embryos, the SPC6 transcript was detected in the developing kidneys/adrenals from day 13.5 of gestation onwards and the level of expression was relatively constant through to the newborn stage. SPC6 expression in the embryonic brain and heart appeared to be transient, initially expressed at day 13.5 in both types of tissues but then showed a decline in signal intensity at day 15.5 in the brain and day 17.5 in the heart. Expression of SPC6 was again detected in the brain of the newborn mouse. The SPC6 transcript was not detected in embryonic liver at any of the stages examined. In the embryonic tissues that expressed SPC6, the signal representing the SPC6-A isoform is more intense than the larger SPC6-B species except in the kidneys/adrenals,

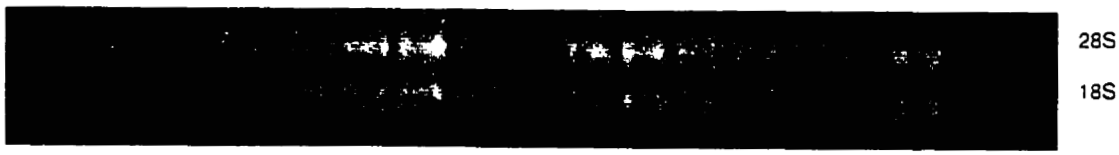
Figure 8: Analysis of SPC6 Gene Expression in Embryonic Tissues

A) Northern blot analysis was carried out on aliquots (~10 μg) of total RNA extracted from various embryonic tissues. RNA from whole embryos (WE) was obtained at days 8.5, 11.5, and 13.5 post-coitum. RNA from brain, heart, kidneys/adrenals, and liver were obtained at days 13.5, 15.5, 17.5, and 19.5/20.5 (newborn stage) post-coitum. The RNA was blotted onto Hybond NX membrane and hybridized with a ^{32}P-labeled SPC6 probe. Autoradiographic exposure time was 3 days at -70°C. The upper band on the autoradiographic film represents the 6 kb SPC6-B transcript and the lower band represents the 3 kb SPC6-A transcript. Abbreviations: B - brain, H - heart, K - kidneys/adrenals, and L - liver. B) The bottom panel shows the 18S and 28S rRNA stained with ethidium bromide.

A.


E 8.5 WE
E11.5 WE
E13.5 WE
E13.5
E15.5
E17.5
newborn
B H K L B H K L B H K L B H K L
SPC6-B
6 kb
SPC6-A
3 kb

B.


28S
18S

which expressed both isoforms more or less equally (Figure 8). The difference in expression pattern was unlikely to be due to unequal loading of RNA because 28S and 18S rRNA was detectable by ethidium bromide staining in all lanes of the formaldehyde-agarose gel. This experiment was repeated using two independent sets of material and identical results were obtained in each case.

3.3 Characterization of SPC6 Expression in the Decidua and Uterus During Pregnancy

3.3.1 Northern Blot Analysis

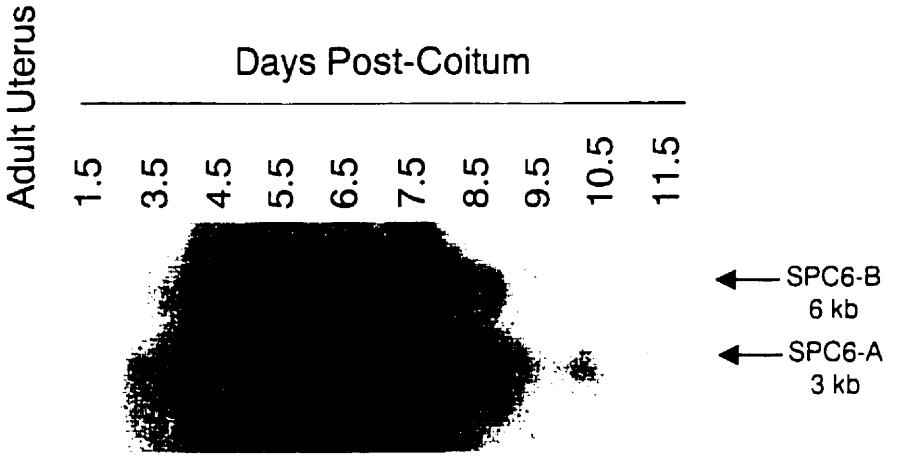
To determine the temporal expression pattern of the SPC6 mRNA during mouse pregnancy, Northern blot analysis was performed on RNA extracted from decidual and uterine tissue obtained from pregnant mice 1.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5 days post-coitum. The adult uterus from a non-pregnant mouse was included to demonstrate the basal level of expression of SPC6.

From Northern blot analysis, a marked increase of SPC6 expression was observed beginning at day 4.5 of pregnancy through to day 8.5 (Figure 9). After day 8.5, SPC6 transcripts were undetectable on the autoradiography film. This decline was unlikely to be due to differences in sample loading since the intensity of ethidium bromide stained 18S and 28S rRNA in the 9.5 day slot is higher than that of the 8.5 day sample where the hybridization signal is still quite strong. In the decidua and uterus during pregnancy, the smaller SPC6-A (3 kb) transcript was more abundant than the larger SPC6-B (6 kb) transcript at all stages

Figure 9: Northern Blot Analysis of SPC6 Gene Expression in the Decidua During Pregnancy

A) Northern blot analysis was carried out on aliquots (~20 μg) of total RNA extracted from the decidual and uterine tissue of pregnant mice at days 1.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5 post-coitum. The decidua were not dissected free of uterine tissue, and included both implantation and non-implantation sites. Normal adult uterus was obtained from a non-pregnant female. The RNA was blotted onto Hybond NX membrane, and hybridized with a ^{32}P-labeled 2.8 kb fragment of pSPC6. Autoradiographic exposure time was 3 days at -70°C. B) The bottom panel shows the ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane.

A.


Adult Uterus
Days Post-Coitum
1.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
SPC6-B
6 kb
SPC6-A
3 kb

B.


28S
18S

examined (Figure 9), and the difference in the proportion of each isoform became more remarkable on day 8.5. The predominance of the SPC6-A transcript at all time points examined during pregnancy corresponds to previous data that the SPC6-A isoform is the dominant species expressed in the uterus. This experiment was repeated on four different occasions and the results were highly reproducible.

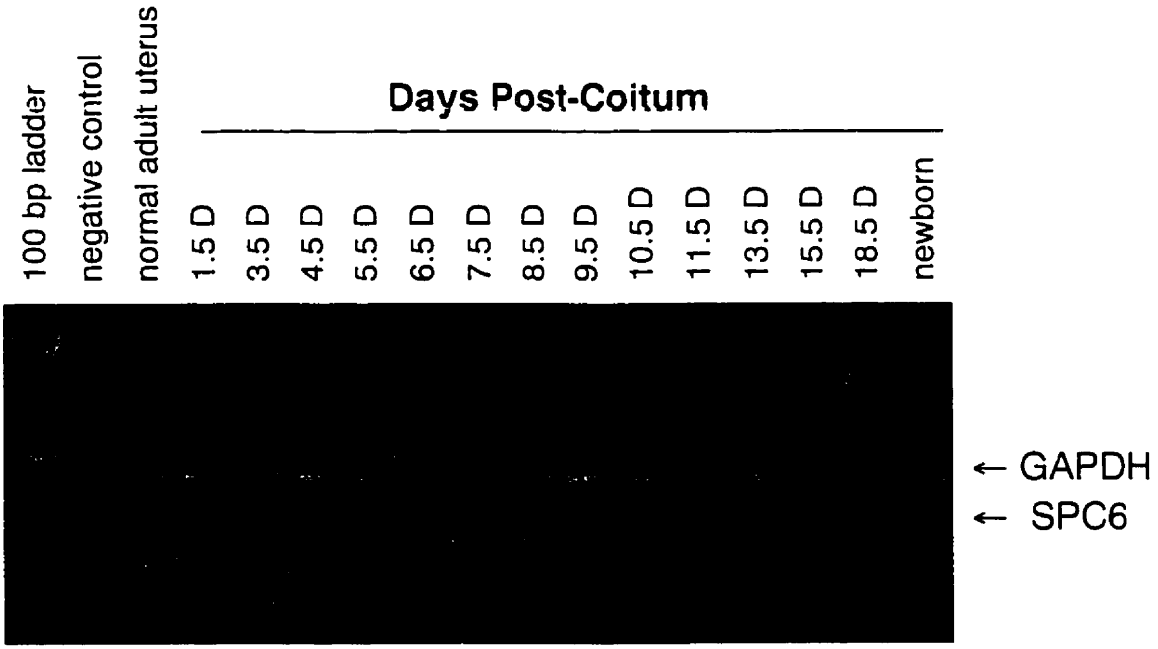
3.3.2 RT-PCR Analysis

To further characterize SPC6 mRNA expression in the decidua and uterus during pregnancy, RT-PCR was employed as an additional technique for rapid and sensitive detection of these transcripts. The SPC6 primers used for the RT-PCR experiments do not distinguish between the SPC6-A and the SPC6-B isoforms because the primers were constructed in a common region of both SPC6-A and SPC6-B. Therefore, the single 300 bp band represents the combined expression of both the SPC6-A and SPC6-B transcripts.

Upon resolution of the PCR reaction products, two DNA bands were observed (Figure 10). The larger band, 450 bp in size, corresponds to a region of the ubiquitously expressed GAPDH transcript and the smaller band, 300 bp in length, is derived from a segment of the SPC6 transcript. The GAPDH (450 bp) band was observed to be relatively constant from one stage to the next. The SPC6 (300 bp) band, on the other hand, exhibited a pattern in which the intensity of the signal began to increase in RNA samples from day 1.5 onwards, reached maximum intensity at day 6.5 and began to decline thereafter (Figure 10). The increase in the intensity of the ethidium bromide stained DNA band corresponds

Figure 10: RT-PCR Analysis of SPC6 Gene Expression in the Decidua During Pregnancy

Total RNA was extracted from the maternal decidua and uterus during various stages of pregnancy and was subjected to reverse transcription. Total RNA was obtained from decidua plus uterus at 1.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 13.5, 15.5, 18.5, and 19.5/20.5 (uterine tissue after birth) post-coitum. The decidua was dissected free of the embryo from day 8.5 onwards. The RNA was resuspended at a concentration of 1 μg/1 μl in DEPC-dH_2O after extraction and was reverse transcribed using random hexamers to make the cDNA. SPC6 and GAPDH specific primers were used in the PCR reaction in which 1 μl of the cDNAs (undiluted RT reaction) per a 25 μl PCR reaction was used for the template. The GAPDH primers served as an internal standard for relative comparison from one sample to the next. The PCR program used for 35 cycles was as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds. Electrophoretic resolution on a 2% agarose gel revealed the 450 bp GAPDH transcript PCR product and a 300 bp SPC6 transcript PCR product. The PCR negative control contained no RNA in the initial reverse transcription reaction.


100 bp ladder
negative control
normal adult uterus
Days Post-Coitum
1.5 D
3.5 D
4.5 D
5.5 D
6.5 D
7.5 D
8.5 D
9.5 D
10.5 D
11.5 D
13.5 D
15.5 D
18.5 D
newborn
← GAPDH
← SPC6

to an increase in the number of SPC6 transcripts present in the decidual RNA. Because RT-PCR is a much more sensitive technique, it was possible to observe an increase of SPC6 expression at an earlier stage and a decline of SPC6 transcripts at a later stage than that which was shown with Northern blots. The identity of the PCR bands was confirmed by sequencing of products in the University of Calgary Core DNA Services Laboratory.

3.4 Expression of SPC6 in Oil-Induced Deciduomas

3.4.1 Northern Blot Analysis

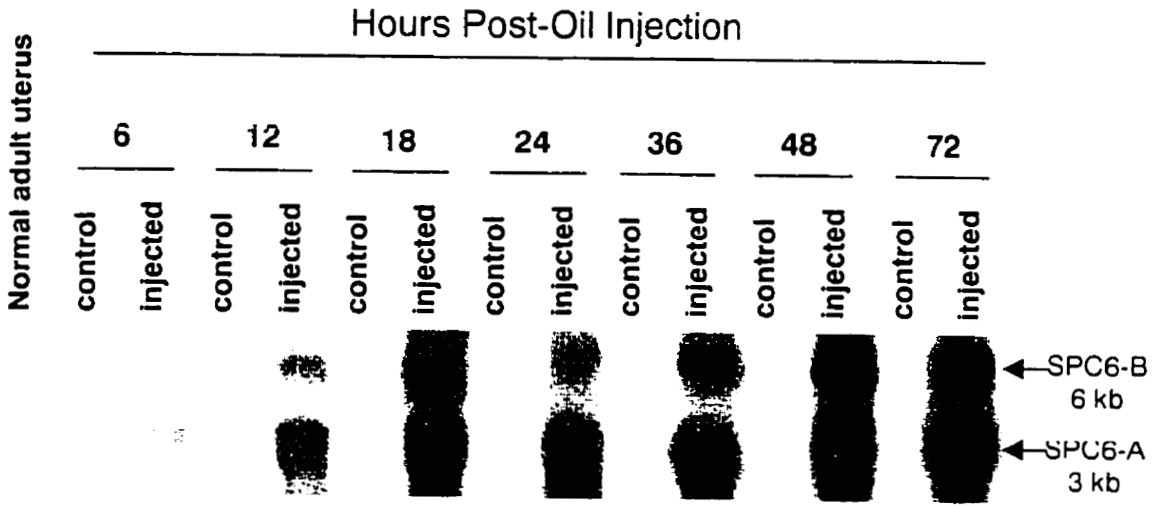
To characterize whether SPC6 expression in the pregnant mouse uterus depended on the embryo as an inducing signal, deciduomas were artificially induced in mice optimally sensitized for decidualization by injection of sesame oil into the lumen of one side of the uterine horn.

In the artificially decidualized uterus, a slight increase in the abundance of SPC6 transcripts was detectable as early as 6 hours after the oil injection (Figure 11). The level of SPC6 mRNA continued to rise and became increasingly abundant as time progressed. The last time point, at 72 hours after oil injection, showed the highest level of SPC6 transcript and demonstrated that this increase in the abundance of transcripts was able to be maintained for at least 3 days duration. Little or no SPC6 transcript was detectable in the non-injected horn at any time point examined even though total RNA loaded in each lane was detectable by ethidium bromide staining (Figure 11). In oil-induced deciduomas, although the SPC6-B transcript was evident at the same time that SPC6-A was

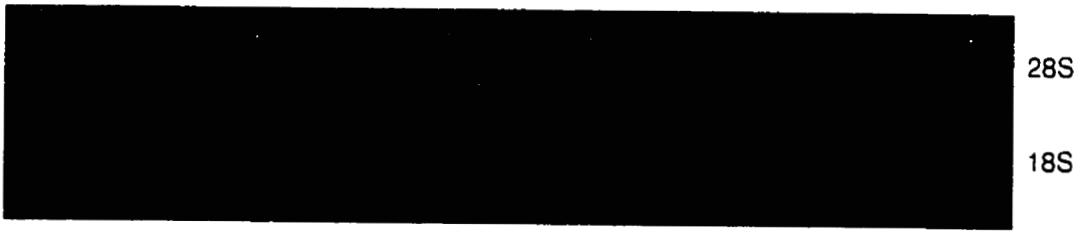
Figure 11: Northern Analysis of SPC6 Transcripts in Artificially Induced Deciduomas.

A) Total RNA was extracted from both injected and uninjected horns of the uterus at 6, 12, 18, 24, 36, 48, and 72 hours post-injection with sesame seed oil (deciduogenic stimulus). Oil was injected into only one side of the uterine horns (labeled as "injected') and the non-injected side served as the control horn (labeled as "control"). RNA from normal, non-pregnant, adult uterus was used as a standard for this experiment. The RNA was extracted using Trizol® , resuspended in DEPC-dH_2O, and ~10 μg was applied to denaturing formaldehyde agarose gels to resolve SPC6 transcripts electrophoretically. The RNA was then immobilized onto Hybond NX and probed with a ^{32}P-labeled fragment of the pSPC6 cDNA. Autoradiographic exposure time was 2 days at -70°C. The upper band on the autoradiographic film represents the 6 kb SPC6-B transcript and the lower band represents the 3 kb SPC6-A transcript. B) The bottom panel shows the ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane.

A.


Hours Post-Oil Injection
Normal adult uterus
6
12
18
24
36
48
72
control
injected
SPC6-B
6 kb
SPC6-A
3 kb

B.


28S
18S

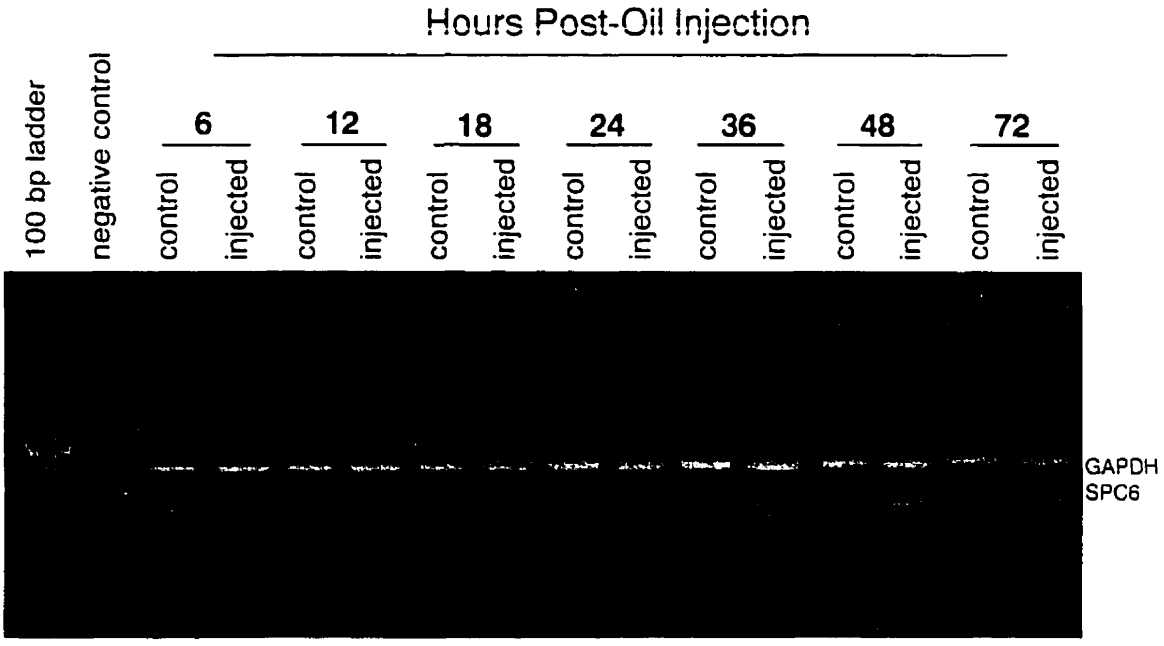
initially expressed, the SPC6-B isoform was never expressed as abundantly as the SPC6-A isoform at any time points examined. This once again demonstrated that SPC6-A is the dominant isoform in the uterus. This experiment was repeated on three different occasions, using three different sets of RNA, and identical results were obtained in each experiment.

3.4.2 RT-PCR Analysis

To support the findings from the Northern blot analysis, the more sensitive method of RT-PCR was also performed on the RNA obtained as described above. Upon resolution of the PCR products in agarose gel, the relative intensity of the 300 bp SPC6 band in comparison to the 450 bp GAPDH standard was observed to be detectable and constant up to 12 hours post-oil injection. However, from 18 hours post-oil injection onwards, the relative intensity of the SPC6 PCR product from RNA from the injected horn was significantly greater than that in the non-injected horn. Although SPC6 transcripts were still detectable in the non-injected horn, the intensity of the band was less than half of the signal intensity for the injected horn (Figure 12). The decrease in signal intensity in the non-injected horn was not due to a lack of cDNA template because as the GAPDH band revealed, a more-or-less equal amount of template was supplied in each of the PCR reactions. Overall, as seen in the Northern blots analysis, SPC6 expression sharply increased with the induction of the decidualization process in the uterus.

Figure 12: RT-PCR Analysis of SPC6 Gene Expression in Artificially Induced Deciduomas

Total RNA was extracted from artificially induced deciduomas at various hours after the injection with the deciduogenic substance (sesame seed oil) into the lumen of the uterus and was subjected to reverse transcription. Oil was injected into one side of the uterine horns (labeled as "injected') and the non-injected side served as the control horn (labeled as "control"). RNA from normal adult uterus was used to demonstrate the basal level of expression of SPC6. The decidua was dissected free of the embryo from day 8.5 onwards. After reverse transcription, PCR was performed on the cDNA using the SPC6 primers and GAPDH primers. The PCR program used for 35 cycles was as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds. The amplification using GAPDH primers served as a standard for the PCR reaction. Electrophoretic resolution on a 2% agarose gel revealed the 450 bp GAPDH transcript PCR product and a 300 bp SPC6 transcript PCR product. The negative control contained no RNA in the initial reverse transcription reaction.


Hours Post-Oil Injection
100 bp ladder
negative control
6
12
18
24
36
48
72
control
injected
GAPDH
SPC6

3.4.3 In situ Hybridization Analysis

To characterize the spatial expression of the SPC6 gene in oil-induced deciduomas, *in situ* hybridization was carried out on 10 μm sections of oil-induced deciduoma tissue. For the hybridization, DIG-labeled sense (negative control) and anti-sense riboprobes were transcribed from pBS.SPC6-5'. Hybridization using the anti-sense probe to a section of the injected horn revealed the expression of SPC6 transcripts in the differentiated, decidual cells surrounding the lumen of the uterus (Figure 13). Staining was not detected in the uterine muscle layer nor in the mesometrial tissue. On a section of the control horn, the side which did not receive an oil injection, SPC6 transcripts were not detectable in high abundance in the undifferentiated, stromal cells. In additional control experiments, *in situ* hybridization using DIG-labeled sense riboprobes was performed on adjacent sections of the deciduomas. The sense probes did not hybridize to any parts of the deciduoma. The pattern of expression of SPC6 in oil-induced deciduomas is remarkably similar to that observed for TIMP-3 in similar material (Bany and Schultz, 2000). Previous studies have also demonstrated an overlap of sites of expression of SPC6 and TIMP-3 in the decidual zone during implantation in the mouse uterus (Rancourt and Rancourt, 1997).

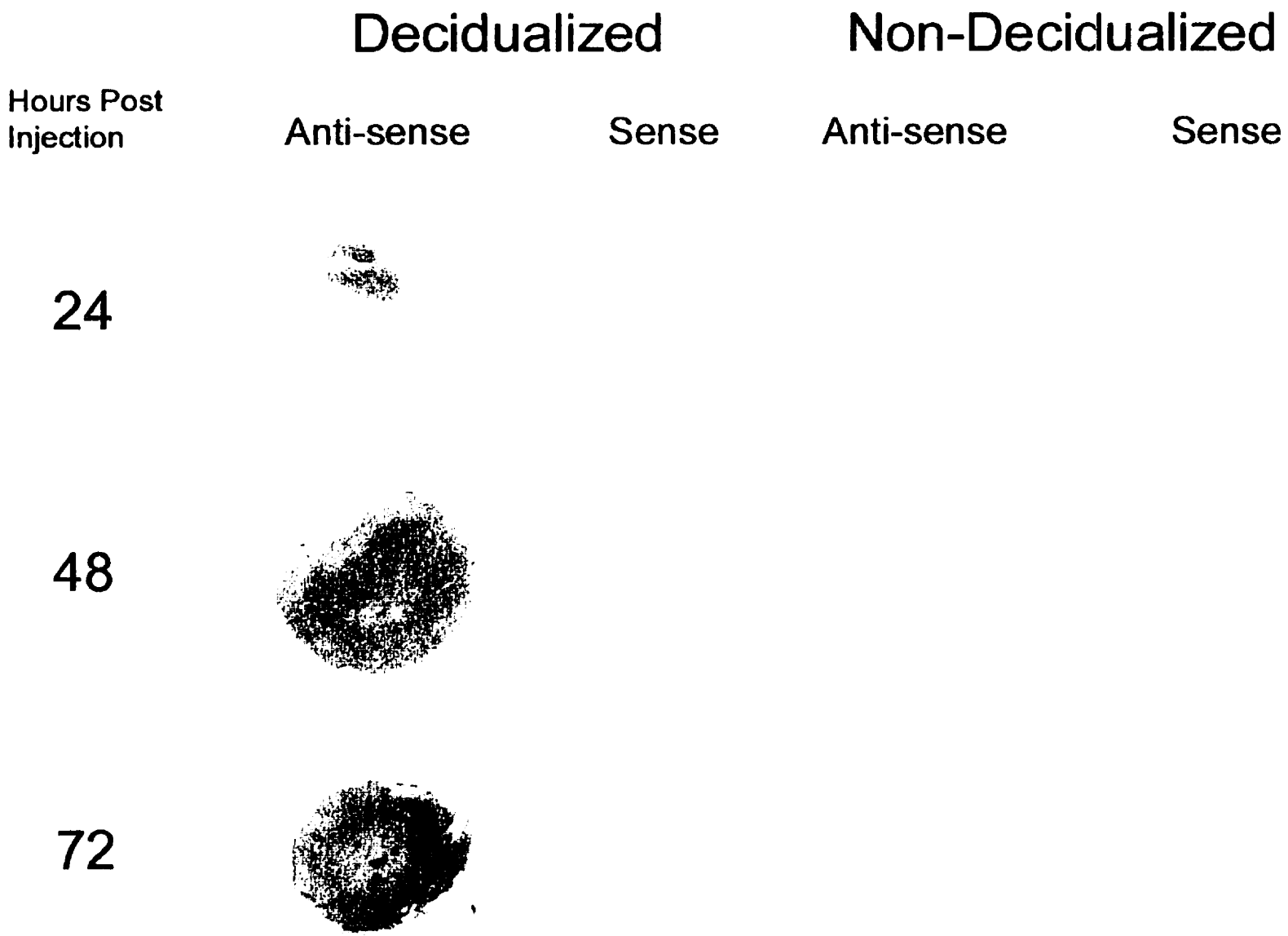
3.5 Expression of TGF- β1 and TGF-β2 in the Implantation Site

3.5.1 RT-PCR Analysis

An experiment was conducted to verify previous reports that TGF-β1 and

Figure 13: *In situ* Hybridization Analysis of SPC6 in Artificially Induced Deciduomas

Artificial deciduomas were induced in mice according to the protocol outlined in Materials and Methods and the mice were sacrificed 24, 48, and 72 hours after injection with the deciduogenic substance. Oil was injected into one side of the uterine horns (labeled as "decidualized") and the non-injected side served as the control horn (labeled as "non-decidualized"). The deciduomas were embedded in paraffin wax, 10 μm thick sections were obtained and attached to silanized slides. The deciduoma sections were subjected to *in situ* hybridization using anti-sense and sense DIG-labeled SPC6 probes according to the *in situ* hybridization protocol outlined in Materials and Methods. After hybridization and color development, the sections were counter-stained with eosin.


Decidualized
Non-Decidualized
Hours Post Injection
Anti-sense
Sense
Anti-sense
Sense
24
48
72

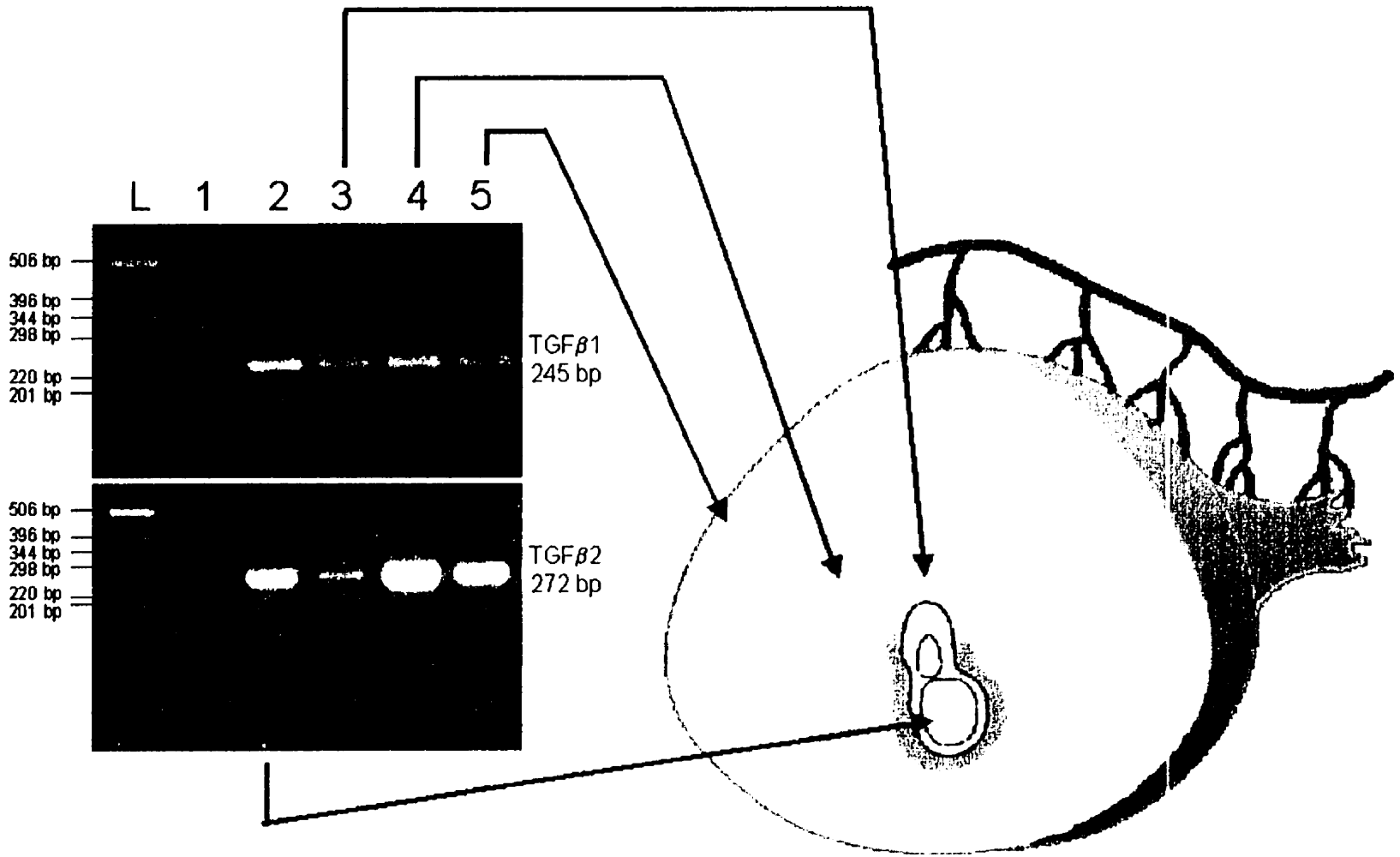
TGF-β2 transcripts are expressed in the uterus during the peri-implantation period. RNA was isolated from the embryo, ectoplacental cone, decidua, and uterus on day 7.5 post-coitum. RT-PCR was carried out using primers for TGF-β1 and TGF-β2 transcripts. It was observed that both TGF-β1 and TGF-β2 PCR products were detectable in RNA derived from all parts of the uterus surrounding the embryo as well as the embryo proper (Figure 14). Identity of the PCR products was verified, as before, by DNA sequencing as well as by restriction enzyme digestion.

3.5.2 Western Blot Analysis

Experiments were also carried out to examine if it was feasible to observe TGF-β1 processing from its latent to active form in protein extracts from the implantation and non-implantation sites of uteri from day 5.5 to 7.5 of pregnancy. TGF-β1 molecules, like other members of the TGF-β superfamily, are synthesized as inactive precursors that are homodimeric molecules linked by disulfide bonds with a molecular weight of approximately 45 kDa (Miller *et al.*, 1990). Proteolytic cleavage produces the active TGF-β1 molecule from the C-terminus of the complex that has a molecular weight of about 12.5 kDa. Thus, if western blot methods could be used to detect both the precursor and active forms of TGF-β1 within decidual extracts, then treatment of decidual stromal cells with inhibitors of processing proteinases like SPC6 should lead to accumulation of the precursor form and reduction in the smaller active form. To test this possibility, commercial antibody to TGF-β1 (Santa Cruz Biotechnology, Inc.) and

Figure 14: RT-PCR Analysis of TGF-β Expression in the Decidua and Embryo

Total RNA was extracted from various parts of the decidua and uterus as indicated and subjected to reverse transcription. PCR was programmed for 35 cycles as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The upper panel shows the PCR with TGF-β1 primers and the bottom panel is PCR performed with TGF-β2 primers. Lane L indicates the 1 kb DNA ladder (Gibco-BRL). The negative control is shown in lane 1. For each lane, the RNA was obtained from the various parts of the decidua as indicated by the arrows: lane 2 - the embryo proper, lane 3 - ectoplacental cone, lane 4 - maternal decidua, and lane 5 - uterus.


L 1 2 3 4 5
506 bp
396 bp
344 bp
298 bp
220 bp
201 bp
TGFβ1
245 bp
506 bp
396 bp
344 bp
298 bp
220 bp
201 bp
TGFβ2
272 bp

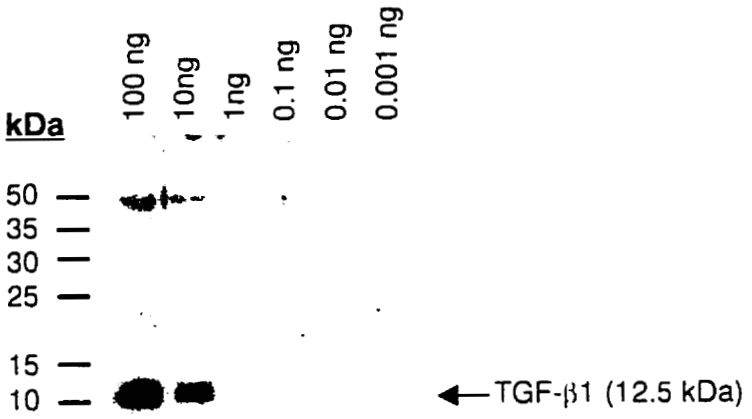
purified TGF-β1 protein (active 12.5 kDa component, Santa Cruz Biotechnology Inc.) were subjected to western blot experiments along with decidual protein extracts. The control TGF-β1 peptide was diluted in serial dilutions ranging from 0 to 10^{-5} and was resolved on a 15% SDS-PAGE gel. Anti-TGFβ1 antibody was added at a dilution of 1/2500.

Chemiluminescent detection of 100 ng of the TGF-β1 protein was achieved after a 2 minute exposure to Kodak BML film, but a 30 minute exposure time was necessary for detection of 10 ng of the protein (Figure 15, panel A). An experiment using implantation and non-implantation site (E5.5, E6.5,and E7.5) protein extracts was conducted in parallel with the control experiment. It was found that even at 2 minutes of exposure time to film, extraneous background signals in the decidua protein extracts were already beginning to appear (Figure 15, panel B). At 30 minutes of exposure time, the background signals from the decidua proteins were extremely intense, but signal for the mature (12.5 kDa) form of TGF-β1 was not detectable within the decidual proteins. If the latent 45 kDa form was present, it was masked by strong non-specific staining from other proteins in the 50 kDa region of the gel. Therefore, although TGF-β1 mRNA was detectable by RT-PCR, detection of the TGF-β1 protein in decidual protein extracts using Western blots was not possible due to the low level of sensitivity of the TGF-β1 antibody and the low amount of TGF-β1 protein present in the decidual tissue.

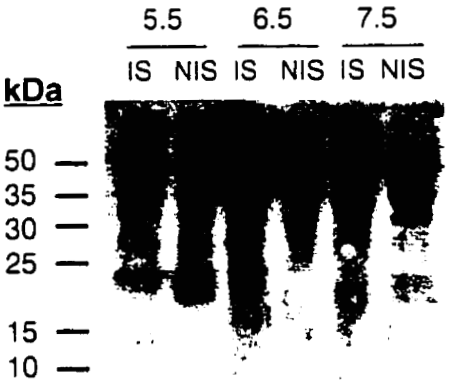
Figure 15: Western Blot Assay for TGF-β1 Protein in the Decidua.

A) Western blot of purified TGF-β1 protein (12.5 kDa) at different dilutions, ranging from 100 ng to 0.001 ng to test the sensitivity of the TGF-β1 antibody. TGF-β1 protein was resolved on a 15% SDS-PAGE gel by electrophoresis and immobilized onto Hybond P membrane. ECL was applied to the membrane after incubation with the primary and secondary antibodies for chemiluminescence detection. An exposure time of 30 minutes was necessary for detection of 10 ng of pure TGF-β1 protein. The bottom panel B) shows the experiment performed in parallel using proteins extracted from pregnant decidua. The protein extract was obtained from implantation (IS) and non-implantation sites (NIS) and 10 μg of the extract was loaded into each lane of the 15% SDS-PAGE gel for electrophoresis. The migration position of the molecular weight markers is shown on the left side of both panels.

A.


100 ng
10ng
1ng
0.1 ng
0.01 ng
0.001 ng
kDa
50
35
30
25
15
10
TGF-β1 (12.5 kDa)

B.


5.5
6.5
7.5
IS NIS IS NIS IS NIS
kDa
50
35
30
25
15
10

3.6 Expression of TIMP-3 in Adult Mouse Tissues

The previous experiments demonstrated that the plan to test the hypothesis that SPC6 might cleave and activate latent TGF-β1 molecules in the uterus could not be carried out through direct analysis because of the limitation of sensitivity of the western blot assay for TGF-β1. Thus, an alternative, indirect approach was taken that was based on the previous observations that TGF-β1 molecules can induce the expression of TIMP-3 molecules in many cells types including the decidualizing uterus (Leco *et al.*, 1992; Bany and Schultz, 2000). The rationale herein was that if SPC6 inhibitors could be used to interfere with TGF-β1 processing, a downstream effect might be a reduction in TIMP-3 expression in decidua or implantation sites in the mouse uterus. Thus, these experiments began with a re-examination of TIMP-3 expression in the decidualizing uterus followed by experiments to detect changes in TIMP-3 expression in endometrial stromal cell cultures treated with an SPC6 inhibitor.

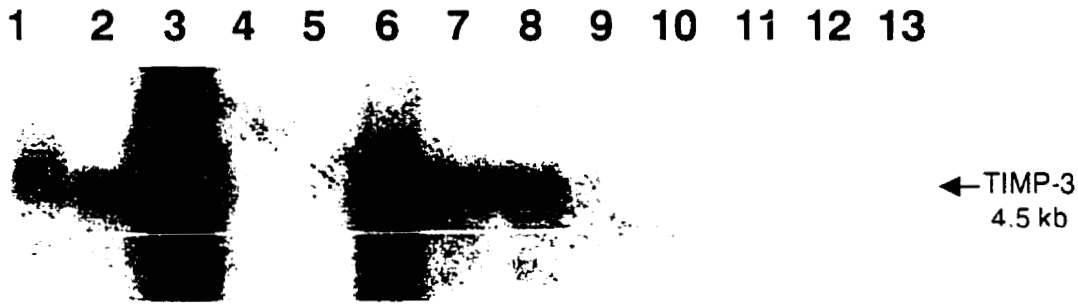
Northern hybridization analysis of adult mouse tissues indicated high levels of TIMP-3 expression in the kidneys/adrenals and lungs (Figure 16). TIMP-3 expression was also found in lower abundance in the brain, heart, and ovaries (from both pregnant and non-pregnant animals). RNA was loaded more or less equally in all the wells of the formaldehyde-agarose gel, therefore, absence of TIMP-3 signal was unlikely to be due to a lack of RNA in the gel. To determine the expression pattern of the TIMP-3 gene during normal mouse pregnancy, RNA was isolated from the decidua and uterus of mice at

Figure 16: Analysis of TIMP-3 Gene Expression in Adult Mouse Tissues and During Pregnancy

A) Northern blot analysis was carried out on aliquots (~10 μg) of total RNA extracted from various adult tissues, blotted onto Hybond NX membrane. The probe used for detection of TIMP-3 mRNA molecules in Northern blots of mouse RNA samples was a 760 bp fragment of the original TIMP-3 cDNA clone (Leco *et al.*, 1994) that was subcloned into pBluescript (kindly provided as a gift by B. Bany). The 760 bp fragment was excised by restriction digestion with *EcoRI* and *PstI* and was radiolabeled with ^{32}P by the random priming method, and was used for hybridization to Northern blots of various RNA samples. Autoradiography film exposure time was 2 days at -70°C. The band shown in the diagram is the 4.5 kb TIMP-3 transcript. Lanes: 1 - brain tissue, 2 - heart, 3 - kidneys/adrenals, 4 - large intestines, 5 - liver, 6 - lungs, 7 - normal ovaries, 8 - ovaries during pregnancy, 9 - skeletal muscle, 10 - small intestines, 11 - spleen, 12 - stomach, 13 - virgin uterus. Panel B) shows the ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane. C) Northern blot analysis performed on 10 μg of total RNA extracted from the uterus and decidua during pregnancy. The RNA was blotted onto Hybond NX membrane and hybridized with a ^{32}P-labeled TIMP-3 probe. Autoradiographic exposure time was 2 days at -70°C. The band shown in the figure is the 4.5 kb TIMP-3 transcript. (Abbreviation: UT = normal adult uterus) D) Ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane. E) RT-PCR was

performed on the RNA obtained from the decidua and uterus at various stages of pregnancy. For RT-PCR, the RNA was reverse transcribed and 1 μl of the cDNA from the reverse transcription reaction was subject to PCR amplification using TIMP-3 and GAPDH primers. The PCR program used is as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR products for TIMP-3 (244 bp) and GAPDH (450 bp) were resolved on a 2% agarose gel and visualized under UV-illumination. (Abbreviation: UT = normal adult uterus)

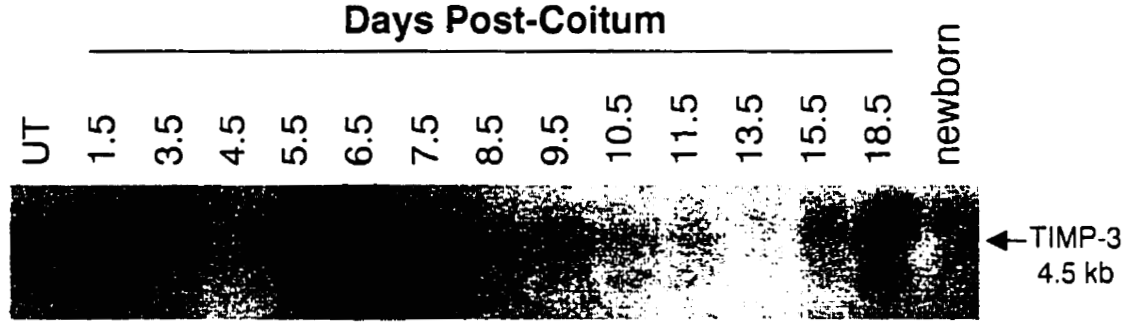
A.


1 2 3 4 5 6 7 8 9 10 11 12 13
TIMP-3
4.5 kb

B.


28S
18S

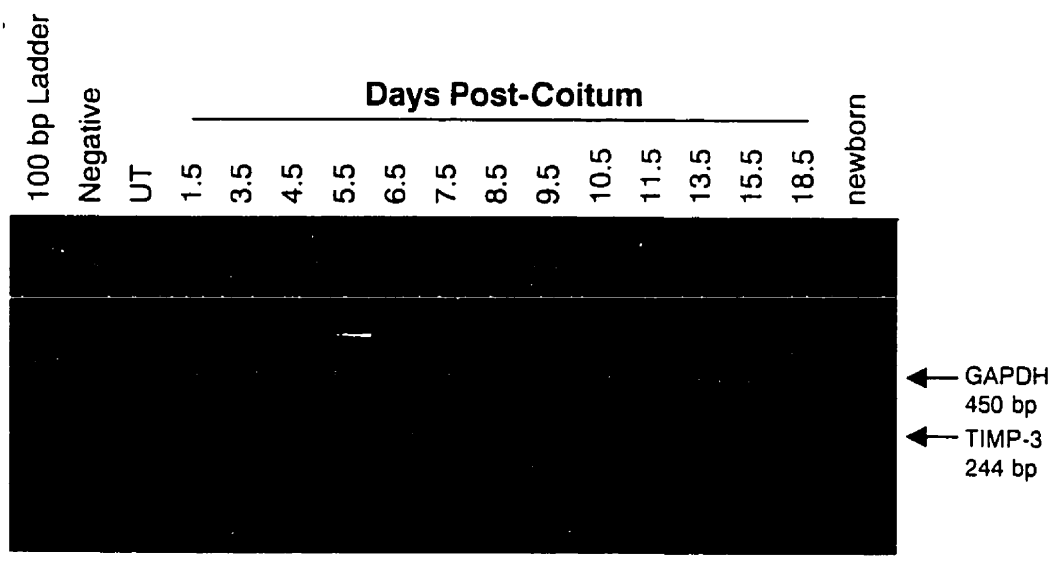
C.


Days Post-Coitum
UT
1.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
13.5
15.5
18.5
newborn
TIMP-3
4.5 kb

D.


28S
18S

E


100 bp Ladder
Negative
UT
Days Post-Coitum
1.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
10.5
11.5
13.5
15.5
18.5
newborn
GAPDH
450 bp
TIMP-3
244 bp

various times during gestation. Hybridization of the TIMP-3 probe to immobilized total RNA revealed a strong signal from days 5.5 to 7.5 of gestation (Figure 16). The level of TIMP-3 transcripts was maximal at day 6.5 post-coitum as demonstrated by the sharp increase in signal intensity (Figure 16). An equal amount of RNA was loaded into each lane of the formaldehyde-agarose gel, thus, the difference in signal intensity detected in the Northern blot was likely to reflect the relative abundance of the TIMP-3 transcript.

To confirm the findings of the Northern blot, RT-PCR was performed on the decidual and uterine RNA isolated during pregnancy. The results of the RT-PCR demonstrated that TIMP-3 transcripts are present throughout all stages of pregnancy. Corresponding to the Northern blot analysis, the expression of TIMP-3 was found to be the strongest at day 6.5 of gestation, represented as the brightest band when stained with ethidium bromide and UV-illuminated (Figure 16). GAPDH primers were used as a standard to demonstrate that a more or less equal amount of template cDNA was supplied to each PCR reaction.

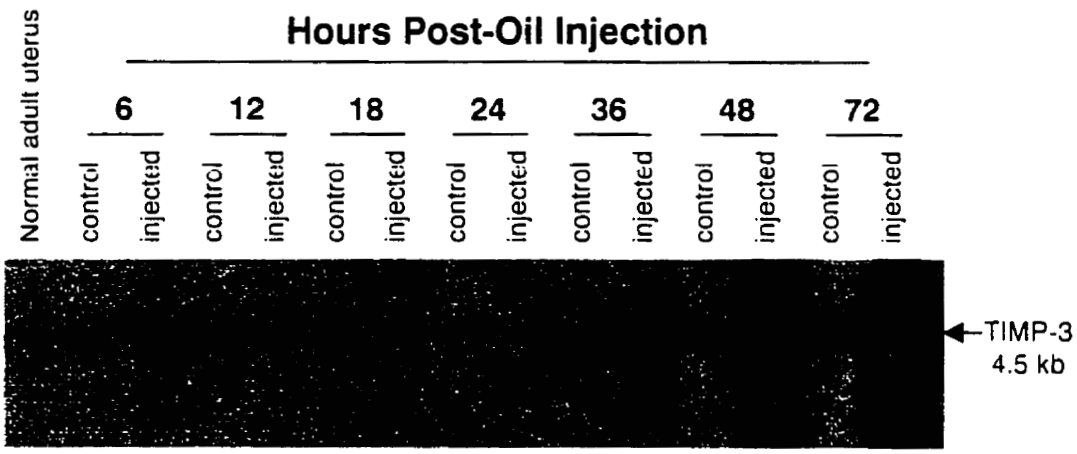
3.7 Expression of TIMP-3 in Oil-Induced Deciduomas

For this experiment, the blot that contained the immobilized deciduoma RNA, previously probed with SPC6, was reused. As shown in the autoradiography after hybridization with the TIMP-3 probe, TIMP-3 steady state mRNA level was observed to remain at a relatively low and constant level of expression for the first 24 hours after injection with the deciduogenic (sesame seed oil) substance (Figure 17), although TIMP-3 expression was consistently

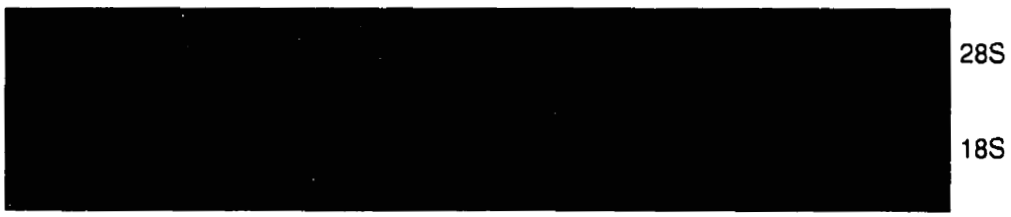
Figure 17: Northern Analysis of TIMP-3 Expression in Oil-Induced Deciduomas

A) Northern blot analysis was carried out on 10 μg of total RNA extracted from artificially induced deciduomas at various hours after the injection with the deciduogenic substance (sesame seed oil) into the lumen of the uterus. Oil was injected into one side of the uterine horns (labeled as "injected') and the non-injected side served as the control horn (labeled as "control"). RNA from normal adult uterus was used to demonstrate the basal level of expression of SPC6. The RNA was blotted onto Hybond NX membrane and hybridized with a ^{32}P-labeled TIMP-3 probe. Autoradiographic exposure time was 2 days at -70°C. The band on the autoradiographic film represents the 4.5 kb TIMP-3 transcript. B) The bottom panel shows the ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane.

A.


Hours Post-Oil Injection
6
12
18
24
36
48
72
Normal adult uterus
control
injected
TIMP-3
4.5 kb

B.


28S
18S

greater in the injected horn than in the non-injected horn. At 48 hours post-oil injection, however, a great increase in the amount of TIMP-3 transcripts was observed in the injected horn but not in the control horn, and this increase in expression was maintained even at 72 hours post-oil injection (Figure 17). The difference in signal intensity does not reflect lack of RNA loaded in each lane because 18S and 28S rRNA staining revealed that even though less RNA was in the 24 - 72 hour lanes, the signals appeared the most intense for those stages.

3.8 Expression of TIMP-3 in Endometrial Stromal Cell Cultures After Addition of SPC Inhibitor

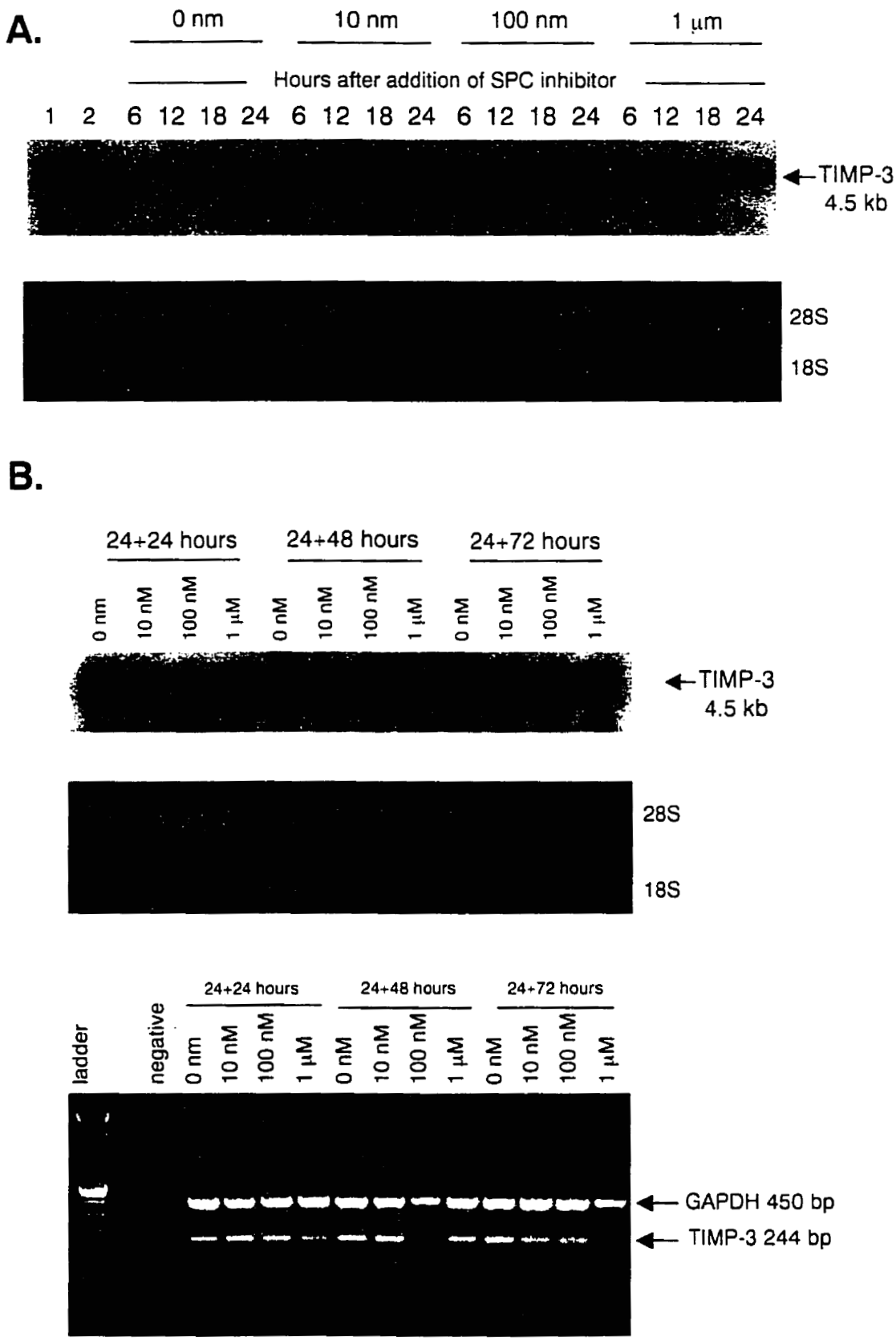
To test whether TIMP-3 gene expression could be affected by the inhibition of SPC6 activity, an *in vitro* assay was designed whereby the inhibitor to SPC6, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (K_i=0.1 nM; Bachem Biosciences Inc.), was added to the endometrial stromal cell culture media. Endometrial stromal cell cultures were set up as described in Materials and Methods and serum was added to the culture media after the attachment period.

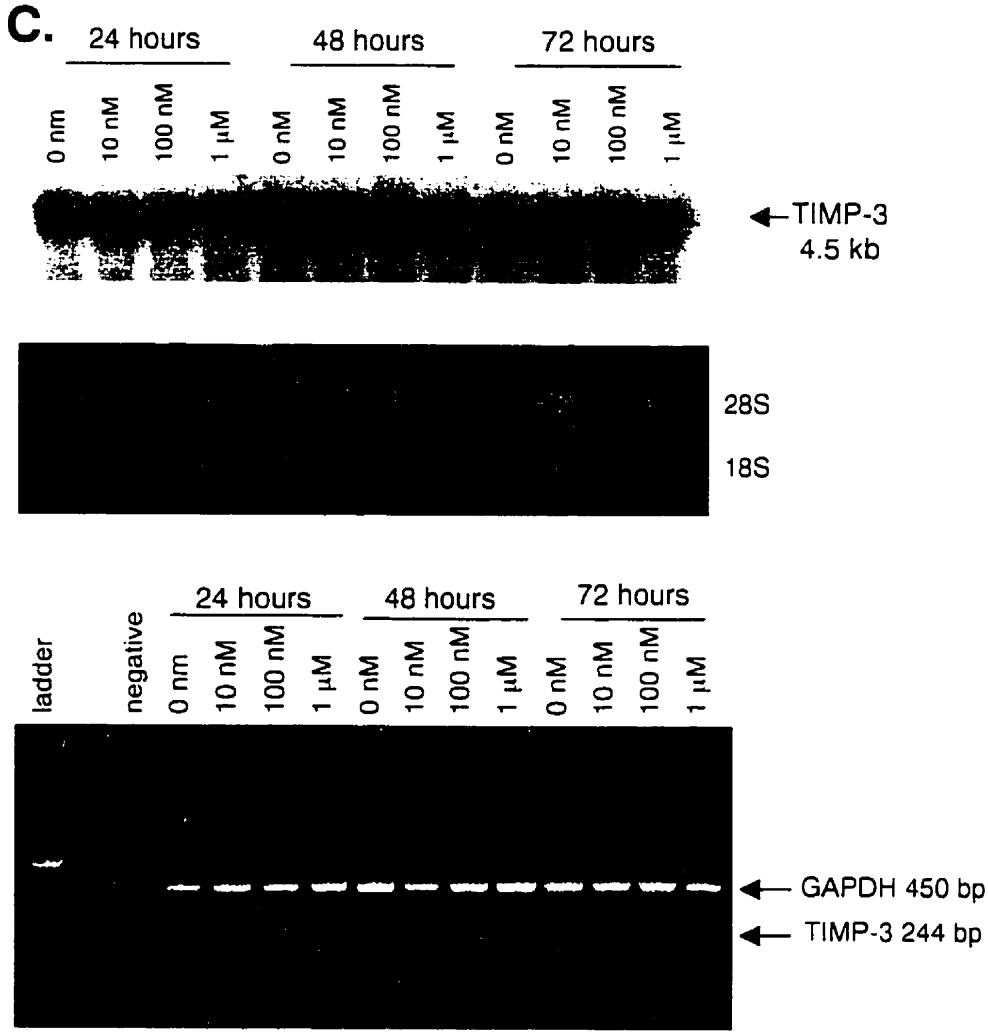
In the first experiment, the cells were cultured in serum for 24 hours prior to the addition of the inhibitor. The cells were then harvested for RNA extraction at 6, 12, 18, and 24 hours after the initial addition of 0 nM, 10 nM, 100 nM, and 1 μM of the SPC inhibitor. Northern blot analysis of the endometrial stromal cell culture RNA for TIMP-3 gene expression revealed virtually constant levels of TIMP-3 transcripts expressed up to 24 hours after the addition of 0 nM, 10 nM, 100 nM, and 1 μM of the SPC inhibitor (Figure 18). The level of TIMP-3

Figure 18: Tissue Culture and Inhibitor Experiments

A) Endometrial stromal cells were cultured in serum for the first 24 hours before the addition of the SPC inhibitor. The inhibitor was added in concentrations of 10 nM, 100 nM, and 1 μM. RNA was extracted from $3x10^6$ cells at the various times indicated. Lane 1 represents RNA extracted at time 0, before the addition of serum to the media. Lane 2 is RNA from cells at 24 hours after the initial addition of serum and before addition of the inhibitor. The RNA was immobilized onto Hybond NX membrane and hybridized with a ^{32}P-labeled TIMP-3 probe. Exposure time was 2 days at -70°C. The panel directly below shows the ethidium bromide staining pattern of 28S and 18S rRNA on formaldehyde-agarose gel prior to the transfer onto Hybond NX membrane. B) SPC inhibitor was added to the culture media 24 hours after the cells were cultured in serum. Inhibitor was added in concentrations of 0 nM, 10 nM, 100 nM, and 1 μM. Fresh media and inhibitor were fed to the cells every 24 hours thereafter. The cells were cultured over a period of 72 hours after the initial addition of the inhibitor. At 24, 48, and 72 hours of culture, the cells were washed in PBS and harvested for RNA extraction. For Northern blot analysis, 10 μg of the RNA was resolved on a 1% formaldehyde-agarose gel and immobilized onto Hybond NX. The blot was probed with a ^{32}P-labeled TIMP-3 probe and exposed to autoradiography film for 48 hours at -70°C. The topmost panel shows the results of the Northern blot. Ethidium bromide staining of the 18S and 28S rRNA bands in the middle panel demonstrates equal loading of RNA in each lane of the gel. The bottom panel shows the results of the RT-PCR analysis. For RT-PCR, the RNA was reverse

transcribed and 1 μl of the cDNA from the reverse transcription reaction was subject to PCR amplification using TIMP-3 and GAPDH primers. The PCR program used is as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR products for TIMP-3 (244 bp) and GAPDH (450 bp) were resolved on a 2% agarose gel and visualized under UV-illumination. C) SPC inhibitor was added to the culture media after an attachment period. Inhibitor was added in concentrations of 0 nM, 10 nM, 100 nM, and 1 μM. Fresh media and inhibitor were fed to the cells every 24 hours thereafter. The cells were cultured over a period of 72 hours after the initial addition of the inhibitor. At 24, 48, and 72 hours of culture, the cells were washed in PBS and harvested for RNA extraction. The Northern blot and RT-PCR analyses were performed as outlined above. The topmost panel shows the results of the Northern blot. The middle panel shows the ethidium bromide staining of the 18S and 28S rRNA bands to demonstrate relative equal loading of RNA in each lane of the gel. The bottom panel shows the results of the RT-PCR analysis.


A.
0 nm
10 nm
100 nm
1 μm
Hours after addition of SPC inhibitor
1 2 6 12 18 24 6 12 18 24 6 12 18 24 6 12 18 24
TIMP-3
4.5 kb
28S
18S
B.
24+24 hours
24+48 hours
24+72 hours
0 nm
10 nM
100 nM
1 μM
0 nM
10 nM
100 nM
1 μM
0 nM
10 nM
100 nM
1 μM
TIMP-3
4.5 kb
28S
18S
ladder
negative
24+24 hours
24+48 hours
24+72 hours
0 nm
10 nM
100 nM
1 μM
0 nM
10 nM
100 nM
1 μM
0 nM
10 nM
100 nM
1 μM
GAPDH 450 bp
TIMP-3 244 bp


C.
24 hours
48 hours
72 hours
0 nm
10 nM
100 nM
1 µM
0 nM
10 nM
100 nM
1 µM
0 nM
10 nM
100 nM
1 µM
TIMP-3
4.5 kb
28S
18S
ladder
negative
24 hours
48 hours
72 hours
0 nm
10 nM
100 nM
1 µM
0 nM
10 nM
100 nM
1 µM
0 nM
10 nM
100 nM
1 µM
GAPDH 450 bp
TIMP-3 244 bp

transcripts did not vary with increasing concentrations of inhibitor nor with increasing length of time of exposure to the inhibitor.

A second series of experiments was carried out to examine whether a longer culture period with the SPC inhibitor in the media would affect TIMP-3 gene expression. In these experiments, two sets of endometrial stromal cell cultures were set up. In the first set of cultures, the cells were cultured in serum for the first 24 hours before treatment with 0 nM, 10 nM, 100 nM, and 1 μM of the SPC inhibitor. These cells were then harvested for RNA extractions at 24, 48, and 72 hours after the addition of the inhibitor. Northern blot analysis of the RNA obtained from this culture demonstrated that TIMP-3 gene expression was not affected by the amount of inhibitor nor the length of time the cells were in culture (Figure 18, panel B). To attempt to verify these results, RT-PCR was performed on the RNA obtained as mentioned above. The RT-PCR analysis showed that a relatively constant level of TIMP-3 gene expression was maintained at a concentration of up to 1 μM of inhibitor and for up to 72 hours after the initial addition (Figure 18, panel B).

For the second set of cultures, the SPC inhibitor was added to the cells directly after the attachment period at concentrations of 0 nM, 10 nM, 100 nM, and 1 μM, and the cells were harvested at 24, 48, and 72 hours after the addition of the inhibitor. Northern blot analysis of these inhibitor-treated cells revealed that TIMP-3 gene expression remained constant throughout culture period and was maintained up to 72 hours after the initial addition of the inhibitor (Figure 18, panel C). Again, to attempt to verify the findings of the Northern blot, RT-PCR

was performed on the RNA obtained as outlined above. The RT-PCR experiment confirmed the above findings that up to 1 μM of inhibitor did not affect TIMP-3 gene expression for at least 72 hours in culture.

Moreover, the effect of a wider range of inhibitor concentration on the expression of the TIMP-3 gene was assessed. RT-PCR analysis demonstrated that addition of the SPC inhibitor at concentrations ranging from 10^{-1} to 10^{5} nM did not appear to affect the level of TIMP-3 expression (data not presented). These results indicate that neither the amount of inhibitor nor the length of the culture period produced any pronounced affect on TIMP-3 gene expression.

In all of the experiments conducted in association with Figure 18, cell cultures were also monitored microscopically for any obvious changes in cell morphology, cell density or overall appearance. In no case was the microscopic appearance of the cultured cells, even at the highest doses of the SPC6 inhibitor used for extended times up to 72 hours, different from control cells not receiving the inhibitor (data not presented).

4. Discussion

Since the discovery of furin in 1990, the family of subtilisin-like proprotein convertases (SPC) have grown to include seven members and the list of candidate proproteins which the SPCs are able to activate is rapidly expanding. The main SPC of interest in this study was SPC6, a member less well characterized than the ubiquitously expressed furin. This study provided an in depth characterization of SPC6 gene expression and endeavored to define a functional role for SPC6 in the process of tissue remodeling during mouse embryo implantation. Experiments were designed to test the hypothesis that in the tissue remodeling process during mouse embryo implantation, SPC6 functions to cleave precursor TGF-β1 protein to generate its active form and that the active TGF-β1 molecule would subsequently exert its effect to influence the expression of the TIMP-3 gene. Results from this study indicate that although the temporal and spatial gene expression profile of SPC6 may indicate a role for this molecule in the mouse embryo implantation process, there is no conclusive evidence to argue that SPC6 is responsible for the processing of the latent TGF-β1 protein to induce the transcription of the TIMP-3 gene.

The distribution profile for each member of the SPC family is distinct and characteristic for each individual member. For SPC6, alternative splicing generates two isoforms, SPC6-A and SPC6-B, that are sorted to different compartments within a cell (De Bie *et al.*, 1996; Seidah and Chretien, 1997; Zhou *et al.*, 1999; Bergeron *et al.*, 2000). SPC6-A is a soluble form sorted to dense core secretory granules which allow the enzyme to interact with many

proproteins that are secreted in the regulated secretory pathway. SPC6-B, however, contains a transmembrane domain and a cytosolic tail which enable it to anchor to membranes of the TGN, and exit via the constitutive secretory pathway. SPC6-B is thereby provided with strategic access to many precursor proteins that move to the cell surface via constitutive vesicles. Differing proportions of each SPC6 species within any given cell type generates functional diversity within a tissue or organ. For example, *in situ* hybridization studies have shown that in the rat brain, SPC1, SPC4, and SPC7 are expressed in both neurons and glial cells but SPC2, SPC3, and SPC6 expression has been found exclusively in neurons (Bergeron *et al.*, 2000). Neurons contain both a regulated and a constitutive secretory pathway, in contrast to glial cells, which only contain a constitutive secretory pathway. These data suggest that SPC2, SPC3, and SPC6 may have specialised functions within the regulated secretory pathway. Likewise, a high proportion of SPC6-A expression in any given tissue may indicate a key role in the processing of particular secreted proproteins that are sorted only to dense core secretory molecules, as opposed to proproteins that are constitutively expressed. Conversely, if a candidate proprotein is known to be found exclusively in the constitutive secretory pathway, SPC6-A activation of this precursor protein can be ruled out.

In adult mice and rats, SPC6-A expression has been found to be widespread in endocrine and non-endocrine tissues, being especially abundant in the intestines and adrenals (Lusson *et al.*, 1993; Nakagawa *et al.*, 1993; Seidah and Chretien, 1994; Bergeron *et al.*, 2000). SPC6-B transcripts are

distributed mainly in the lungs, intestine, and adrenals. From this study, in addition to the tissues mentioned above, the SPC6-B message was also demonstrated to be present in skeletal muscles and SPC6-A mRNA was found in high abundance in the uterine tissue of adult mice (Figure 7). Even though SPC6 transcripts were found in many tissue types, it should be noted that each cell type does not express only one SPC at a time, but express a cocktail of SPCs characteristic of a particular tissue type (Bergeron *et al.*, 2000). SPC6 may be the dominant player in the tissues in which it is found in high abundance, but may act in conjunction with other SPCs to process the melange of precursor proteins specific to each cell type. Moreover, the proportion of each SPC6 isoform in any given cell type may indicate if SPC6 has a general or a regulatory processing role in the particular tissue.

In embryonic tissues, SPC6 was found to be expressed in the kidneys/adrenals throughout all examined stages of development. Transient expression was detected in the brain and heart of the embryo, and the liver was found to be completely devoid of SPC6 transcripts throughout development (Figure 8). The developmental expression profile of SPC6 has been previously studied by Constam *et al.* (1996) and Rancourt and Rancourt (1997), but the observation of SPC6 expression in the developing embryonic brain has not been previously documented. We detected transient expression of SPC6 in the developing brain between days 13.5 to 15.5 of gestation (Figure 8). It is possible that SPC6 may be responsible for the processing of various pro-neuropeptides during mouse brain development. Correspondingly, SPC6-A has been shown to

process the proneuropeptide, pro-neurotensin, *in vitro*, in the rat pheochromocytoma PC12 cell line (Barbero *et al.*, 1998). During embryonic development, the relative proportion of SPC6-A transcript is consistently greater than that of the SPC6-B transcript, except in the kidneys/adrenals. Differences in the distribution of each isoform suggest that the majority of proprotein processing by SPC6 during embryonic development is related to the regulated secretory pathway.

The expression pattern of SPC6 in the decidua and uterus during mouse embryo implantation was examined. In a previous study, SPC6 transcripts were found in the uterus at day 6.5 of mouse pregnancy (Rancourt and Rancourt, 1997). *In situ* hybridization data showed localization of SPC6 transcripts throughout the differentiated decidua in the peri-implantation zone, in the trophoblast cells surrounding the implantation site and in the ectoplacental cone. Northern blot analysis and RT-PCR data from this study demonstrated a profound increase in SPC6 gene expression beginning at day 4.5 post-coitum, coinciding with the embryo implantation process (Figures 9 and 10). During mouse embryo implantation, vast tissue remodeling and vascularization occurs within the uterus and decidua to accommodate the invading embryo (reviewed in Schultz and Edwards, 1997; Rinkenberger *et al.*, 1997). At the completion of the implantation process at around day 8.5 to 9.5 post-coitum, Northern blot and RT-PCR analysis showed the re-establishment of the previous basal level of SPC6 expression (Figures 9 and 10). The temporal and spatial expression patterns of SPC6 imply some important role for the processing enzyme SPC6 in the

implantation process.

SPC6 has not been studied extensively and the list of precursor proteins that SPC6 has been shown to cleave is small. SPC6 has been shown to activate pro-Mullerian substance, to induce cleavage of the extracellular domains of the receptor protein tyrosine phosphatase, and to process pro-neurotensin (Campan *et al.*, 1996; Nachtigal and Ingraham, 1996; Barbero *et al.*, 1998). It is thought that SPCs exhibits redundant functions within the cell because in many tissue types, there is an overlap of expression of different SPC members, and also because in cell lines that lack a certain SPC, the viability of the cells is not compromised. For example, furin, the most extensively studied member of the SPC family, is ubiquitously expressed in all tissues examined (except in the uterus; D. Rancourt, unpublished data) and has been shown to process a wide variety of constitutively secreted proproteins including insulin pro-receptor, pro-von Willebrand factor, pro-β-nerve growth factor, pro-transforming growth factor-β1, and pro-endothelin-1 (Bergeron *et al.*, 2000). But furin is not essential for survival as demonstrated by the furin deficient LoVo cell line in which a complete absence of active furin does not confer lethality to the cells (Dubois *et al.*, 1995). The redundant action of other SPC members may compensate for the absence of furin. Therefore, it is very likely that other less well-studied SPC members may also cleave the same precursor molecules as furin. Thus, precursor TGF-β1 protein may be processed by SPC6 as well.

The temporal and spatial expression pattern of SPC6 in the decidualizing uterus during pregnancy bears a striking resemblance to the expression pattern

of the TIMP-3 gene. TIMP-3 is the major metalloproteinase inhibitor found in the decidualizing uterus during implantation (Alexander *et al.*, 1996; Leco *et al.*, 1996). From day 5.5 to 7.5 of gestation, a dramatic upregulation of TIMP-3 transcripts was demonstrated by Northern blot analysis and by RT-PCR, but by day 8.5, only trace TIMP-3 expression remains (Figure 16). *In situ* hybridization demonstrated localization of TIMP-3 transcripts in maternal decidual cells immediately adjacent to the trophoblast cells of the embryo at day 6.5 post-coitum (Leco *et al.*, 1996).

Another similarity between SPC6 and TIMP-3 expression is found in their expression patterns in oil-induced deciduomas. Northern blot and RT-PCR analysis demonstrated that both SPC6 and TIMP-3 gene expression was intrinsic to the decidualization reaction and was not induced by the embryo (Compare figures 11 and 12 with figure 17). The data obtained revealed that SPC6 and TIMP-3 transcripts were detectable in high abundance in the differentiated decidual cells of artificially induced deciduomas. *In situ* hybridization analysis demonstrated that the SPC6 expression occurred within the differentiated decidual cells of the artificial deciduomas and expression was not detected in the uterine tissue or in the mesometrium (Figure 13). Similarly, TIMP-3 gene expression was also detected in decidual cells surrounding the empty lumen of the uterus (Leco *et al.*, 1996). However, as was seen in the decidua during mouse pregnancy, SPC6 expression was more extensive and encompassed a larger area within the decidualized zone than TIMP-3 gene expression. In the deciduoma, both SPC6 and TIMP-3 gene expression were triggered by the

decidualization reaction and were not dependent upon the presence of a viable embryo. Presently, little is known about how TIMP-3 gene expression is directly regulated *in vivo* but *in vitro* studies have shown that TGF-β1 is capable of mediating TIMP-3 synthesis (Leco *et al.*, 1992; Bany and Schultz, 2000).

A study carried out to investigate TGF-β expression within the decidua crypt demonstrated that TGF-β1 and TGF-β2 transcripts are found in the developing embryo, the ectoplacental cone, the decidual cells surrounding the embryo, and in the uterus (Figure 14). Since TGF-β1 is a candidate molecule for SPC6 processing (Dubois *et al.*, 1995) and it has been shown to induce TIMP-3 gene expression, TGF-β1 seemed to be the logical link between SPC6 proprotein processing and TIMP-3 gene expression. Therefore, a hypothesis was formulated to propose that SPC6 functions to cleave precursor TGF-β1 into its active form, which in turn, acts to regulate TIMP-3 gene expression.

If the hypothesis proved correct, then inhibition of SPC6 activity should lead to the accumulation of TGF-β1 precursors. The general SPC inhibitor, decanoyl-arginine-valine-lysine-arginine-chloromethylketone (Dec-RVKR-CH_2Cl; K_i=0.11 nM) was employed to attempt to assess TGF-β1 processing in the absence of SPC6 activity (Bachem). To assay for TGF-β1 processing, it was initially felt that western blot analysis would suffice. Using western blots, the plan was to assess the relative proportions of the latent TGF-β1 molecule (45 kDa) and the truncated, active form (12.5 kDa) using an antibody which recognized both forms of the protein. The intention of addition of the SPC inhibitor to

endometrial stromal cell cultures was to block the cleavage of precursor TGF-β1 and lead to an accumulation of the 45 kDa TGF-β1 precursor that could be detectable on a western blot. Unfortunately, because TGF-β1 is a potent growth factor expressed in very minute quantities, it was not possible to detect the TGF-β1 protein (either the precursor or the processed form) on western blots. Also, the TGF-β1 antibody used lacked the sensitivity to detect less than 10 ng of the TGF-β1 protein (Figure 15).

Because western blot analysis lacked the sensitivity to assess SPC6 processing of the TGF-β1 precursor, Northern blot and RT-PCR were employed to determine whether the inhibition of SPC6 activity had an effect on TIMP-3 gene expression. If the proposed hypothesis was true, then blocking SPC6 activity would prevent the activation of latent TGF-β1 molecules and result in the decreased production of TIMP-3 transcripts.

Addition of the SPC inhibitor to endometrial stromal cell cultures did not produce any pronounced effect on the viability of cells and the inhibitor was not toxic to the cells even at high concentrations (up to 10 μM was examined) and when applied for long periods of time (up to 72 hours). The cell cultures that were treated with the inhibitor (in concentrations of 10^{-1} to 10^{5} nM) were phenotypically identical to the cultures that were not treated with the inhibitor (data not shown). Northern blot and RT-PCR analysis did not show a decrease in the steady state level of TIMP-3 mRNA even in the presence of up to 10 μM of inhibitor (Figure 18).

There are several explanations as to why an effect was not observed in TIMP-3 expression upon treatment with the SPC inhibitor in the endometrial stromal cell cultures. It was assumed that SPC6 is a secreted protein and processes precursor proteins extracellularly. This assumption may be incorrect because even though SPC6 is sorted to secretory molecules, it may exert its effect on precursor proteins inside these secretory molecules, thereby processing proproteins as they travel to the surface of the cell. This may very well be the case because SPC6-A is the major species found in the uterus and decidua, and this isoform acts in the regulated pathway so it is not constitutively secreted, as opposed to the SPC6-B isoform. Therefore, because the inhibitor was applied to the cell culture media, processing of proproteins taking place inside the cell would not be affected by the extracellular inhibitor. Additionally, SPC6 located inside the cell would not be accessible by the inhibitor and even if inhibition of the less abundant extracellular SPC6-B was successfully achieved, it may not be sufficient to block TGF-β1 processing. In the study by Cui *et al.* (1998) that used the α1-antitrypsin Portland to inhibit the processing of another TGF-β family member, BMP-4, by the SPC convertases, the transcript for the inhibitor was injected into the *Xenopus* oocytes to provide the inhibitor intracellularly.

Another explanation may be a shortcoming in the experimental design. The inhibitor, Dec-RVKR-CH_2Cl, is a peptidyl chloroalkylketone with peptide moieties that mimic the SPC cleavage recognition motif. Dec-RVKR-CH_2Cl is an active site directed irreversible inhibitor with a K_i of 0.11 nM for SPC6 (Jean *et al.*, 1998). The earliest time point in which the cells were harvested for RNA

extraction was 6 hours after the addition of the inhibitor to the culture media. It was possible that within the 6 hour time frame, excess inhibitor was eliminated by enzymatic digestion by the proteinases present in the serum and new molecules of SPC6 were generated. The cells were harvested at 6 hour intervals because in a previous study, it was found that the greatest increase in TIMP-3 gene expression was detected about 12-18 hours after the addition of pure TGF-β1 to endometrial stromal cell cultures (B. Bany, unpublished data).

Another possible reason the inhibitor did not have an effect on TIMP-3 expression may be because TGF-β1 is not the target of SPC6 action in endometrial stromal cells and SPC6 may exert its action on other growth factors or proproteins. For example, the uterus also expresses other TGF-β family members like TGF-β2 and TGF-β3 (reviewed in Roelen and Mummery, 2000). Many genes are upregulated at the time of implantation and tissue remodeling. Because many precursor molecules possess the SPC recognition motif, it is also very possible that SPC6 participates in the tissue remodeling process by activating other types of proproteins such as pro-hormones or pro-MMPs.

Although the SPC inhibitor did not appear to have an effect on the expression of the TIMP-3 gene *in vitro*, SPCs may still play a vital role in the decidualization and tissue remodeling process in the uterus during mouse embryo implantation. Additional experimentation is needed to further dissect the role of SPC6 during embryo implantation. The generation of a conditional SPC6 mutant mouse to determine if fertility is affected might provide insight into SPC6

action. A null SPC6 knock-out mutant may not be very useful because SPC6 expression during development and during pregnancy is tightly regulated, therefore, null mutants may be embryonic lethal. Also, in conditional mutants, the expression profile of different genes expressed during implantation can be assessed to determine if the expression of any specific gene is altered by the absence of SPC6. In addition, new technologies such as microarrays or gene-chip technology and advances in proteomics will allow for the rapid evaluation of the functions of the SPC6 enzyme in living cells.

Implantation and pregnancy are processes that are wholly dependent on hormones secreted by the endocrine system. It is possible that SPC6 may exert its effect on precursor hormone molecules or hormone receptors. It would be useful to study if the activation of such prohormones or their receptors is affected by the presence of the SPC inhibitor. It is very likely that SPC6 may be the receiver and facilitator of hormonal messages communicated by the endocrine system.

In vitro and *in vivo* cleavage assays may provide valuable insight to the identity of proproteins that SPC6 is capable of processing. Previously, *in vivo* cleavage assays have primarily been done with the intensely studied furin to demonstrate the ability of furin to activate latent proteins such as pro-insulin receptor, pro-TGF-β1, pro-von Willebrand factor, pro-β-nerve growth factor, and pro-endothelin-1 (reviewed in Bergeron *et al.*, 2000).

Overall, the temporal and spatial expression pattern of SPC6 does suggest a role for this converting enzyme in the process of mouse embryo

implantation. Our studies did not provide any conclusive evidence for its function in the tissue remodeling process and, thus, the exact role of SPC6 in implantation remains to be established. Through the course of this study, extensive expression data on SPC6 in adult and embryonic tissues, and during mouse pregnancy has been acquired. This information will help to direct future studies in development, reproduction, and fertility.

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