ADAMTS EXPRESSION IN THE HUMAN DECIDUA OF EARLY PREGNANCY

BY

YORK HUNT NG

B.S., OKLAHOMA CITY UNIVERSITY, USA, 2001

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

THE FACULTY OF GRADUATE STUDIES
THE FACULTY OF MEDICINE
DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
PROGRAM OF REPRODUCTIVE AND DEVELOPMENTAL SCIENCES

WE ACCEPT THIS THESIS AS CONFIRMING TO THE REQUIRED STANDARD

THE UNIVERSITY OF BRITISH COLUMBIA

AUGUST 2004

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JULY 2004

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Degree: Master of Science Reproductive and Developmental Sciences

Department of Obstetrics and Gynaecology

The University of British Columbia

Vancouver, BC Canada
ABSTRACT

The spatio-temporal expression of interleukin (IL)-1β and transforming growth factor (TGF)-β1 in the human endometrium suggests that these two cytokines act in concert with gonadal steroids to create a uterine environment that is capable of supporting a viable pregnancy. The highly regulated series of developmental events that culminates in the terminal differentiation of the stromal cells into deciduas in preparation for implantation is mediated by alterations in the composition of the endometrial extracellular matrix (ECM). Consequently, matrix metalloproteinases have been assigned central roles in implantation and placentation. I hypothesized that members of a novel family of metalloproteinases, the ADAMTS, may mediate certain proteolytic events that are operative at the maternal-fetal interface. A comprehensive survey of the ADAMTS subtypes present in first trimester human decidual tissue was performed. In addition, I have examined the ability of IL-1β and TGF-β1 to regulate the mRNA and protein levels of one of these endometrial ADAMTS subtypes, ADAMTS-1, in primary cultures of stromal cells isolated from first trimester decidua. The mRNA transcripts encoding ADAMTS-1, -2, -3, -4, -5 (-11), -6, -7, -8, -9, -10 and -12 are present in first trimester human deciduas. ADAMTS-1 mRNA transcript and protein expression were detected in all of the decidual stromal cell cultures using quantitative-competitive-polymerase chain reaction (QC-PCR) and Western blotting strategies. IL-β1 (0-1000 IU) increased ADAMTS-1 mRNA and protein levels in decidual stromal cell cultures in a dose-dependent manner. Significantly increased ADAMTS-1 mRNA and protein levels were also detected in cells cultured for 24 hours in the presence of a fixed amount of IL-1β
(100 IU), with maximum levels being observed after 48 hours. In contrast, increasing concentrations of TGF-β1 (0-10 ng) decreased ADAMTS-1 mRNA and protein levels in these cells in a dose-dependent manner. Furthermore, a fixed concentration of TGF-β1 (5 ng) effected a significant time-dependent decrease in ADAMTS-1 mRNA and protein levels in decidual stromal cells that was readily apparent at 24 hours and continued to decline until the study was terminated at 48 hours. Collectively, these studies indicate that IL-1β and TGF-β1 have differential effects on ADAMTS-1 mRNA and protein levels in decidual stromal cells in vitro. These results suggest that cytokine-mediated regulation of ADAMTS-1 expression, and potentially of other ADAMTS family members, may be important in stromal cell differentiation that is essential for decidual function during early pregnancy.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin and Metalloproteinase with ThromboSpondin repeats</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EVT</td>
<td>Extravillous cytotrophoblast</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HIP</td>
<td>Heparin/ heparan sulfate-interacting protein</td>
</tr>
<tr>
<td>HS</td>
<td>Heparin/ heparan sulfate</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IVF-ET</td>
<td>In vitro fertilization-embryo transfer</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK 1/2</td>
<td>MAPK/ERK kinase ½</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane-type metalloproteinases</td>
</tr>
<tr>
<td>MRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>P4</td>
<td>Pregestosterone</td>
</tr>
<tr>
<td>PAI</td>
<td>Pasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>QC-PCR</td>
<td>Quantitative competitive polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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Finally, I wish to thank my parents and my sister for their unconditional support and encouragement throughout my studies. Here, I dedicate this thesis to both my parents and my sister, by expressing my deepest appreciation to them.
PART I. OVERVIEW

1.1: Background

The establishment of a successful pregnancy is dependent upon the coordinated development of the implanting embryo and the maternal endometrium (Tabibzadeh et al., 1995; Paria et al., 2002). In particular, the endometrium must have undergone a highly regulated series of morphogenetic events in preparation for pregnancy. The blastocyst, in turn, must have attained the ability to interact with the diverse cell types that constitute the endometrium and subsequently form a functional placenta (Graham et al., 1992; Paradinas et al., 1995).

The endometrium is only receptive to the implanting embryo during a defined period of the menstrual cycle, known as the "window of implantation" (Hertig et al., 1956; Nikas et al., 1999; Wilcox et al., 1988). Desynchrony in the spatial or molecular development of the maternal and/or the fetal compartment often results in spontaneous abortion, or in less severe cases, defects in the formation and organization of the placenta that often compromise continuation of pregnancy to term (Paria et al., 2000, 2002; Kao et al., 2002). A better understanding of the cellular mechanisms underlying the tissue remodeling events that occur in the endometrium in preparation for the implanting embryo will allow us to delineate the events involved in the successful establishment and maintenance of pregnancy.
The ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin repeats) are a novel family of extracellular proteases found in both mammals and invertebrates (Tang et al., 2001). One of the best characterized members of this gene family is ADAMTS-1, initially identified as an inflammation-associated gene. Gene knockout studies have subsequently highlighted the important roles that ADAMTS-1 play in tissue morphogenesis and fetal growth. In addition, adult female mice that are null-mutant for this gene exhibited reduced fertility owing to diminished ovarian and/or uterine function (Shindo et al., 2000).

The proteolytic degradation and/or activation of distinct extracellular matrix (ECM) components of the decidua are essential for the establishment and maintenance of human pregnancy (Graham et al., 1992). This progressive developmental process is mediated by the spatio-temporal expression of several cytokines, growth factors and their mutual receptors in decidual cells and/or the trophoblast cells of the implanting embryo (Graham et al., 1992), which in turn, regulate the localized activity of matrix metalloproteinase (MMP) and the urokinase plasminogen activator (uPA) systems at the maternal-fetal interface (Huang et al., 1998; Karmakar et al., 2002). Collectively, these observations have led to embryonic implantation being described as an inflammatory-type response.

Interleukin (IL-1β) is a major pro-inflammatory cytokine that has been associated with the development of an uterine environment that is capable of supporting pregnancy (Simon et al., 1994). In particular, the endometrial expression levels of this cytokine increase during the secretory phase of the menstrual cycle (Huang et al., 1998). This is the time
period when decidualisation and embryonic implantation is initiated. IL-1β level peaks during the first trimester of pregnancy when ECM remodeling of the decidua is at its greatest, until a subsequent decrease as gestation proceeds (Simon et al., 1994). Furthermore, decreased expression levels of IL-1β and its receptor in the secretory endometrium have been associated with unexplained fertility and habitual abortion (Wolff et al., 2000). The biological actions of IL-1β on the endometrium are believed to be mediated, at least in part, via its ability to increase the proteolytic activities of the MMP/TIMP and/or uPA/PA-1 systems expressed in the endometrial stroma and/or in the invading trophoblasts, both of which contribute to the remodeling of this dynamic tissue (Shimonovitz et al., 1996; Huang et al., 1998; Chung et al., 2001).

Similarly, the anti-inflammatory growth factor, TGF-β1 is a potent regulator of the MMP and uPA-mediated endometrial tissue remodeling events underlying pregnancy (Huang et al., 1998). In particular, TGF-β1 has been shown to play a key role in the morphological and functional differentiation of the decidua and in reducing the invasive capacity of extravillous cytotrophoblasts, particularly during the first trimester of pregnancy when maximum levels of this growth factor are expressed in both the maternal and fetal cellular compartments (Lala et al., 1990; Graham et al., 1991). In contrast to IL-1β, TGF-β1 has been shown to decrease MMP and uPA activity in primary cultures of human endometrial stromal cells and extravillous cytotrophoblasts (Roberts et al., 1988; Lala et al., 1990; Graham et al., 1997; Huang et al., 1998). Taken together, these observations suggest that IL-1β and TGF-β1 balance the interplay between the proteolytic mechanisms operative at the maternal-fetal interface.
1.2: Cyclic Remodelling of the Human Endometrium

The stromal and epithelial cells of the human endometrium undergo cyclic proliferation, differentiation and shedding in response to the gonadal steroids, progesterone (P4) and 17β-estradiol (E2) (Noyes et al., 1950). Following menstruation, the endometrium regenerates under the influence of E2 to produce a dense cellular stroma containing narrow tubular glands and small blood vessels. Instantaneously after ovulation, the effects of P4 on epithelial cell morphology can be observed with larger gland profiles and the emergence of basal glycogen masses in these endometrial cells. In contrast, there is little alteration in the histology of the endometrial stromal or vascular cells at this stage of the menstrual cycle. If fertilization occurs, embryonic implantation occurs in the midsecretory phase of the cycle. This phase is therefore a critical nodal point which demonstrates that when an embryo is present, P4 levels will continue to rise, leading to decidualisation of the stroma. On the other hand, in the absence of pregnancy, P4 levels will drop to produce a late secretory phenotype, followed by menstrual shedding.

The putative “window of implantation” in the human is believed to span cycle days 20-24 and involves the luminal epithelium and subsequently endometrial stroma (Hertig et al., 1956; Nikas et al., 1999; Wilcox et al., 1988). This receptive period is associated with distinct molecular and morphological changes in the luminal epithelium of the endometrium. In particular, epithelial dome-like structures (pinopodes), that are believed to mediate the attachment of the embryo to the luminal epithelium, emerge at the implantation site (Lindenberg et al., 1991).
The expression of a number of molecules in this endometrial cell layer including H-type 1 antigen, mucins, heparan sulfate proteoglycan, carbohydrate epitopes, integrin subunits (particulary \( \alpha\nu\beta3 \) and \( \alpha4\beta1 \)) and the trophin-bystin/tastin complex has also been found to be temporally regulated in this endometrial cell layer within and framing the "window of implantation" (Aplin et al., 1995; Suzuki et al., 1999; Lessey et al., 2000, Kao et al., 2002). The mid secretory stroma also demonstrates histological alterations that characterize the initial cascade of differentiative events leading to decidualisation (Noyes et al., 1950). Focal areas of edema become visible in which the density of stromal cells is reduced. As a consequence, blood vessels in these areas are more apparent, even though no overt vascular differentiation is yet evident. Other areas of the stroma remain densely populated with elongated mesenchymal cells. As in other phases of the menstrual cycle, but now more obvious, the periglandular stroma contains a layer of flattened cells in close apposition to the epithelial basement membrane.

In the late secretory phase, the areas of edematous stroma become more widespread, though more densely cellular areas also occur. At this time, vascular differentiation occurs to produce prominent spiral arterioles surrounded by a cuff of pseudo-decidual cells, which are enlarged stromal cells that bear a resemblance to the decidual cells of pregnancy.

Decidualisation, which begins in the secretory phase and continues into early pregnancy in humans, incorporates the morphological and biochemical differentiation of the endometrial stroma. Decidualisation is believed to be a critical event in the development of an uterine environment that is capable of fulfilling regulating embryonic
implantation (Noyes et al., 1950; Kearns et al., 1983). This highly regulated series of developmental events involves the remodeling of the stromal/decidual cell ECM. Morphological decidualisation is expressed histologically by a change to a polyhedral cell shape with an increase in cell size, and ultrastructurally by an extensive development of the organelles involved in protein synthesis and secretion, and by the appearance of gap junctions and desmosomes (Kearns et al., 1983; Wynn et al., 1974; Lawn 1971). Functionally, decidualisation precedes the onset of insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin (PRL) secretions (Kearns et al., 1983; Lala et al., 1984). A significant population of bone-marrow derived cells amounting to over 40% of the total and comprising large granular lymphocytes (LGLs), macrophages and to a lesser extent, T cells also now present (Starkey et al., 1988; Bulmer et al., 1990). The LGLs are believed to develop from a smaller population of precursor cells present in the endometrial stroma during the secretory phase of the menstrual cycle. Close intercellular associations are often seen between these bone marrow-derived cells and resident decidual cells (Aplin et al., 1988). Mast cells have also been detected in the human decidua (Marx et al., 1999).

The diverse populations of cells that compose the decidua allow this dynamic tissue to fulfill paracrine, nutritional, immunoregulatory functions throughout pregnancy (Kearns et al., 1983). Additionally, the decidua is believed to play a central role in the regulation of embryo implantation and in the maintenance of pregnancy through control of trophoblast invasion into the underlying maternal tissues and vasculature throughout early pregnancy (Pijnenborg et al., 1980). The depth of trophoblast invasion is correctly controlled by the
decidua. Errors have extreme consequences on the health of both fetus and the mother (Cross et al., 1994; Paria et al., 2000;).

1.3: **Cellular mechanisms underlying Implantation**

The highly regulated series of developmental processes that occur in the endometrium in preparation for pregnancy are believed to be mediated, at least in part, by alterations in the composition of the endometrial extracellular matrix (ECM) during the menstrual cycle, the breakdown of these matrices during implantation, and the spatiotemporal expression of cell surface receptors that modulate cell-cell or cell-matrix interactions (Tabibzadeh et al., 1995; MacCalman et al., 1998).

1.3.1: **Extracellular matrix deposition**

The ECM is a complex cellular product comprising glycoproteins, collagens, glycosaminoglycans and proteoglycans as major structural and functional components. The proteoglycan superfamily consists of three families: the basement membrane proteoglycans perlecan and agrin; the bamacanhe hyalectans comprising versican, aggrecan, neurocan and brevican; and the leucine-rich proteoglycans comprising decorin, and biglycan. Collectively, proteoglycans are involved in tissue arrangement and organization, with individual proteoglycan molecules performing distinct functions in these processes. For example, versican has been implicated in the regulation of cell migration and developing tissue pattern formation (Perissinotto et al., 2000; Iozzo et al., 2001).
Regeneration of the endometrium during the proliferative phase of the menstrual cycle involves the deposition of a scaffolding of ECM (Aplin et al., 1988; Mylona et al., 1995; Church et al., 1995).

The undifferentiated stroma produces an ECM of classic mesenchymal composition. Collagens I, III, V, and VI and fibronectin (Fn) have all been shown to be present. Also, there are periglandular deposits of tenascin that emerge to reflect the proliferative state of the epithelial compartment (Vollmer et al., 1990). The epithelium and blood vessels are surrounded by basement membranes containing laminin, collagen type IV and heparan sulfate proteoglycan (HSPG). Ovulation has little effect on the composition of the stromal or vascular ECM although collagen deposited into the ECM is organized into fibril bundles that form an anatomising network in the intercellular spaces.

However, changes in the ECM occur with the transition from undifferentiated stroma to decidua (Wynn et al., 1974; Wewer et al., 1985; Kislaus et al., 1987; Ruck et al., 1994). The decidual ECM lacks the bundles of uniform-diameter parallel fibrils found in the intercellular spaces of the endometrial stroma. Fibril diameters and orientations are variable and fibrils are sparsely distributed, though the major collagen types I, III, and V and fibronectin are still present. Type VI collagen is now absent. The decidual cells encapsulate themselves in a pericellular basal lamina through which pedicels protrude. The pedicels contain secretory granules that are probably involved in the secretion of basement membrane components (Kislaus et al., 1986).
The decidual cell basement membrane is composed of laminins 2 and 4, type IV collagen, heparan sulphate proteoglycan (HSPG), and BM-40 (Wewer et al., 1985; Faber et al., 1986; Church et al., 1996). Therefore, the differentiation of endometrial stromal ECM presents two contrasting molecular paradigms. The first is the selective removal during decidualisation of collagen VI, a structural component that plays a key role in the integration and structural stabilisation of tissue architecture, perhaps by cross-linking the major scaffolding elements of the endometrial ECM during the proliferative phase. The focal loss of collagen VI in the endometrial stroma during the mid-secretory phase may mediate, at least in part, the reduction in cellular density and increased edema associated with this stage of the menstrual cycle (Aplin et al., 1988). In addition, the loss of collagen VI during decidualisation may help promote cellular interaction and/or create an uterine environment into which trophoblast invasion may arise more readily occur (Aplin et al., 1991). The second paradigm is the appearance of laminins (Ln) 2 and 4 in alliance with the differentiating stromal cells (Church et al., 1996). As previous studies have demonstrated that laminin 2 is competent to mediate cell attachment and spreading (Brown et al., 1994), it is tempting to speculate that it may play a role in trophoblast adhesion, migration and/or differentiation during early pregnancy. Similar speculations pertain to the migratory bone marrow-derived cells which are often observed to be attached to the pericellular basal lamina. It is also believed that the decidual basement membrane plays a role in the structural organization and integration of decidual ECM that is required to maintain the developing conceptus, expand as the feto-placental compartment grows and be permeable to macromolecules, such as prolactin, secreted by the decidua and destined for the fetal compartment (Aplin et al., 1988; Ruck et al., 1994).
In addition, large amounts of hyaluronan (HA) are found in the human decidual cell layers (Meinert et al., 2001). Versican, a member of a large aggregating chondroitin sulfate proteoglycan family contains a HA binding region suggesting the possibility of versican present in decidua. However, its presence in the deciduas has not yet been identified.

1.3.2: ECM-degradation-proteinases and their inhibitors

A large part of endometrial remodelling seen in both the proliferative and secretory phases of the menstrual cycle involves the degradation of ECM components, particularly interstitial collagens and basement membranes (Fata et al., 2000; Curry et al., 2001). It is a prerequisite for the gradual remodelling during pregnancy to generate a matrix with suitable mechanical properties (Granstrom et al., 1989, 1991). The decidua of early pregnancy is also subject to further degradation by the invading trophoblasts that have been shown to utilize similar cellular mechanisms for ECM degradation to those observed during tumour cell invasion (Yagel et al., 1988; Strickland et al., 1992; Lala et al., 1996; Bischof et al., 2000). Therefore, proteinases and their associated inhibitors are believed to play a key role in human implantation. Two major classes of proteinases that have been studied in human trophoblast and endometrial cells are plasminogen activators and matrix metalloproteinases.

1.3.2-A: Plasminogen activators and their inhibitors

Plasminogen activators (PA) are substrate-specific serine proteinases that mediate cleavage of plasminogen to plasmin. They exhibit a broad range of serine protease activity
The proteinase activator system includes the urokinase-type plasminogen activator (uPA), the tissue-type PA (tPA), the PA inhibitor-1 and -2 (PAI-1 and PAI-2, respectively) and the uPA receptor.

The expression of uPA and tPA in the human endometrium is temporally regulated during the menstrual cycle with maximum levels being detected during the secretory phase and early pregnancy (Casslen et al., 1983; Koh et al., 1992). Similarly, PAI-1 expression is high during the secretory phase and then declines with the onset of menstruation (Koh et al., 1992). The withdrawal of P4 from the culture medium of endometrial stromal cells increases uPA activity and concomitantly decreases PAI-1 expression levels in these cultures (Schatz et al., 1999). These observations suggest that the uPA system plays key roles in the highly regulated series of remodeling events that occur in the endometrium in preparation for pregnancy.

1.3.2-B: Matrix metalloproteinases and their endogenous inhibitors

The matrix metalloproteinases (MMPs) are a large gene family of zinc-dependent proteinases that mediate a range of tissue remodelling processes (Woessner et al., 1991; Fata et al., 2000). To date, 24 distinct members of the MMP gene family have been identified. These distinct MMP subtypes can be further divided into several subgroups based upon their substrate specificities and/or structural similarities: collagenases (MMP-1, MMP-8, MMP-13); gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-7, MMP-10, MMP-11); membrane-type MMPs (MT-MMP1 through MT-MMP6); and a miscellaneous group that contains MMP-12, and MMP-19 through MMP-26. In addition to the
hydrolysis of distinct ECM components, MMPs have been shown to be capable of cleaving cytokines, chemokines, and cytokine/chemokines ligands (either in soluble form or bound to the cell surface); cell adhesion molecules (cadherins and integrins), their own zymogen forms; and other MMPs and proteinases inhibitors such as serpins (Egebald et al., 2002). Generally, MMPs are synthesized as latent zymogens that must be cleaved in order to become activated. The activity of MMPs can be further regulated by the secretion of specific tissue inhibitors of MMPs (TIMPs).

TIMPs are the major endogenous regulators of MMP proteolytic activity in vivo (Woessner et al., 1991). To date, four homologous TIMP subtypes, TIMP-1, -2, -3, and -4, have been identified. TIMPs are small secreted proteins (21-28 kDa) that form tight, non-covalent bonds with the proteolytic domain of the MMP subtypes with a stoichiometry of 1:1 (Woessner et al., 1991; Egebald et al., 2002). The unique structural properties of TIMP-3 allow it to bind to heparan-sulphate-containing proteoglycans and possibly chondroitin-sulphate-containing proteoglycans in the ECM (Yu et al., 2000). TIMPs also exhibit other biological functions that are independent of their ability to inhibit the proteolytic activity of MMPs. For example, TIMP-1 and -2 have mitogenic effects on a number of cell types (Wang et al., 2002) whereas overexpression of these proteins reduces tumor cell growth (Ikenaka et al., 2003). TIMP-3 has been shown to promote apoptosis in human melanoma and colon carcinoma cells (Smith et al., 1997; Ahonen et al, 2003).

To date, 13 MMP subtypes have been detected in the human endometrium during the menstrual cycle (Fata et al., 2000; Curry et al., 2001; Goffin et al., 2003). The complex
expression patterns observed for each of these endometrial MMP subtypes suggests distinct roles in the development, maintenance and regression of this dynamic tissue. In particular, MMP-7, MMP-11, MMP-26, and MT3-MMP expression levels are high during the proliferative phase in the menstrual cycle and then decrease in the secretory phase. In contrast, MMP-2, MMP-19, MT1-MMP and MT2-MMP are constitutively expressed in the endometrium throughout the menstrual cycle whereas MMP-1, MMP-3, MMP-8, MMP-9, and MMP-12 are only detected in the endometrium during menstruation. MMP-2, MMP-3, and MMP-9 (but not MMP-1 or MMP-7) have been detected in the decidua of early pregnancy, whereas there is only MMP-2 and MMP-9 are expressed in this dynamic tissue at term (Xu et al., 2002).

To date, the cellular localization of only some of these MMP subtypes in the human endometrium has been determined. During the follicular phase, MMP-1, MMP-2, and MMP-3 have been detected in the stroma, MMP-7 and MMP-9 are expressed in glandular epithelium, and MMP-9 is also present in neutrophils and monocytes (Rodgers et al., 1993, 1994; Hampton et al., 1995; Irwin et al., 1996; Jeziorska et al., 1996). In the luteal phase, MMP-3, MMP-10 and MMP-11 are present in the stroma, MMP-7 is present in the glandular epithelium, and MMP-9 is expressed in the glandular epithelium and neutrophils (Rodgers et al., 1994; Irwin et al., 1996; Jeziorska et al., 1996). Within menstrual tissue, MMP-1 and MMP-3 have been detected in stromal cells near blood vessels, MMP-2, MMP-9, MMP-10 and MMP-11 are present in the stroma, MMP-7 is in the glandular epithelium and MMP-9 is detectable in monocytes, neutrophils and macrophages (Rodgers et al., 1993, 1994; Hampton et al., 1995; Marbaix et al., 1995; Kokorine et al., 1996).
The human endometrium has also been shown to constitutively express TIMP-1, TIMP-2, and TIMP-3 whereas the expression of TIMP-4 has not been examined (Fata et al., 2000; Curry et al., 2001; Goffin et al., 2003). In contrast to the MMPs, there appears to be only small fluctuations in the overall expression levels of TIMP-1, TIMP-2 and TIMP-3 in the endometrium during the menstrual cycle. However, a localized increase in TIMP-1 mRNA and protein expression has been detected near small arterioles and capillaries in the secretory endometrium and menstrual tissue suggesting that it may be locally regulated in the endometrial vasculature (Rodgers et al., 1993; Hampton et al., 1994; Salamonsen et al., 1995).

Similarly, TIMP-2 mRNA and protein expression levels are higher in the vasculature than in glandular epithelium, stroma or decidua of early pregnancy (Hampton et al., 1994; Zhang et al., 1999). TIMP-3 expression levels also increase in the predecidual cells of the secretory phase, suggesting that it may serve as a cellular marker of decidualisation and/or play a critical role in regulating trophoblast invasion (Goffin et al., 2003; Zhang et al., 1999). Thus, it appears that the regulation of MMP expression levels in the human endometrium involves large cyclic fluctuations in the epithelial and/or stromal cells of the endometrium and at small localised foci within these cellular compartments which occur thought the menstrual cycle. The activity of endometrial MMPs is counterbalanced by the spatial expression of TIMP levels within the two cellular compartments of this dynamic tissue.

The roles of MMPs and TIMPs in the cyclic remodeling events that occur in the endometrium during each menstrual cycle have been extended to primary cultures of
human endometrial cells. Conditioned media from stromal cells isolated from human endometrial tissues have been shown to contain the latent forms of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-11 and TIMP-1, TIMP-2 and TIMP-3 using zymography and reverse zymography, respectively (Salamonsen et al., 1997). The addition of P4, but not E2, to the culture medium of these primary cell cultures is capable of causing a significant decrease in the levels of these MMP subtypes and concomitant increase in TIMP expression levels (Martelli et al., 1993; Osteen et al., 1994; Higuchi et al., 1995; Marbaix et al., 1996; Schatz et al., 1999). In contrast, the withdrawal of gonadal steroids from the culture medium of endometrial stromal cells allowed to undergo steroid-mediated decidualisation, a culture model system believed to mimic the cellular mechanisms underlying menstruation, resulted in a marked increase in all of the MMP subtypes expressed by endometrial stromal cells, but had no effect on TIMP mRNA or protein expression levels in these primary cultures (Salamonsen et al., 1997).

Although MMP and TIMPs have been assigned key roles in implantation and placentation, mice null-mutant for genes encoding MMPs or TIMPs are fertile (Brown et al., 1994; Fata et al., 2000; Nothnick et al., 2000). These observations suggest that other proteinases mediate or suggest overlapping functions of certain MMPs or TIMPs or compensation, at least in part, the highly regulated series of ECM remodeling events that occur in the endometrium in preparation for the implanting embryo.

1.4: ADAMS
The ADAMs (A Disintegrin And Metalloproteinase) are a gene family of transmembrane proteins that contain a disintegrin and a metalloprotease domain (Graham et al., 1993). Thus, the ADAMs have the potential to act as adhesion molecules and/or proteinases. Two generic functions have been proposed for the ADAM proteases: (1) local activation of signaling pathways by the shedding of cell surface cytokines and growth factors and (2) cell migration/ invasion by the degradation of the ECM (Wolfsberg et al., 1995; Black et al., 1998).

Although five ADAM subtypes (ADAMs-9, -10, 12, 17, and -28) have been shown to act as metalloproteases in vitro, only ADAMS-9, -10, and -17 are known to be catalytically active in vivo. In particular, ADAM-9 is responsible for the shedding of HB-EGF (Heparin Binding Epidermal Growth Factor) from cultured cells (Sclondorff et al., 1999), ADAM-10 acts as a sheddase in the Notch signaling pathway (Blobel et al., 1997; Pan et al., 1997; Izumi et al., 1998; Artavanis et al., 1999) and ADAM-17 is involved in multiple ectodomain-shedding events, most notably the release of Tumor Necrosis Factor (TNF)α (Black et al., 1997; Qi et al., 1999). Several observations also suggest that ADAMs may be involved in cell migration. For example, ADAM-10 and snake venom metalloproteases (SVMPs), the closest relatives of ADAMs, have been shown to cleave purified ECM components (Black et al., 1997; Moss et al., 1997) and/or their receptors (Jeon et al., 1999). ADAM-9 has been shown to promote the migration of fibroblasts in vitro (Millichip et al., 1998) whereas ADAM-13 expression has been detected in cranial neural-crest cells, a highly migratory population of cells in the Xenopus embryo (Kamiguti et al., 1996).
However, there is currently no direct evidence linking ADAM protease activity with the adhesive and migratory behaviour of specific cell populations *in vivo*.

1.5: Regulation of Human Implantation and Placentation

Although it has been well established that E2 and P4 are key regulators of the morphological changes that occur in the human endometrium during the menstrual cycle and pregnancy, there is increasing evidence to suggest that other factors are involved in creating an environment that promotes and/or supports a viable pregnancy. For example, prostaglandins (PGs) are believed to be involved in the initiation and maintenance of stromal cell decidualisation in the rodent and human endometrium *in vitro* and *in vivo* (Kennedy et al., 1982; Frank et al., 1994). In particular, prostaglandin E2 (PGE₂) has been shown to potentiate the stimulatory effects of E2 and P4 on prolactin (PRL) secretion of cultured human endometrial stromal cells. As the glandular epithelium of the human endometrium secretes high levels of PGE₂ during the secretory phase of the menstrual cycle, it has been suggested that this hormone also plays a key role in the differentiation and/or permeability of the vasculature (Psychoyos et al., 1995; Kao et al., 2002).

The spatiotemporal expression of several growth factors and their receptors including epidermal growth factor (EGF), insulin-like growth factor (IGF)-II, interleukin (IL)-1β and transforming growth factor (TGF)-β1 in the human endometrium and placenta (Giudice et al., 1994) suggests that they play a central role in human implantation by regulating the decidualisation of the endometrial stroma and/or the differentiation of trophoblasts along
the invasive or non-invasive pathways in an autocrine and/or paracrine manner. For example, the complete interleukin-1 (IL-1) system, including IL-1β messenger ribonucleic acid (mRNA) expression, IL-1 receptor (IL-1R) type I, and intracellular cell IL-1 receptor antagonist, has been detected in the human endometrium (Huang et al., 1998), with maximum levels being detected in the secretory endometrium and decidua of early pregnancy (Simon et al., 1994). Other evidence also supports a role for the IL-1 system in the morphological and biochemical differentiation of human trophoblasts. In particular, IL-1 secreted by human trophoblasts in vivo and in vitro regulate human Chorionic Gonadotropin (hCG) and PGE₂ secretion in these primary cultures (Yagel et al., 1989; Shimonovitz et al., 1994). IL-1 may also play an intermediary role in trophoblast invasion by regulating trophoblast expression of 92-kDa type IV collagenase (Graham et al., 1991). Similarly, transforming growth factor-β1 (TGF-β1), which is produced by the placenta and decidua in vivo (Graham et al., 1991, 1992, 1993) is capable of reducing proliferation and promoting the aggregation, differentiation, and fusion of these isolated extravillous cytotrophoblasts in vitro (Graham et al., 1992, 1993).

Collectively, these observations not only suggest that multiple factors act in concert to coordinate the morphological and molecular development of the decidua and/or the differentiation of trophoblasts along the invasive or non-invasive pathway, but also that a genetic hierarchy which is capable of controlling the progression of these two inter-related attachment must be operative at the maternal-fetal interface. The identification of one or more key elements involved in the early stages of this regulatory cascade will provide opportunities to develop diagnostic tests for patients with infertility and endometrial
disorders. Understanding these key elements could also lead to the development of targeted drug for treating implantation-based infertility, other endometrial disorders involving altered cellular proliferation and/or differentiation such as cancer, and endometrial-based contraception.

1.6: ADAMTS

Recent cloning studies have identified new members of the ADAM family, known as ADAMTS (A Disintegrin And Metalloprotease with ThromboSpondin motifs-1), in C. elegans, Drosophila and mammals (Kaushal et al., 2000; Tang et al., 2001). ADAMTS are secreted proteins which do not contain the EGF-like, transmembrane and cytoplasmic domains characteristic of other members of the ADAM gene family (Kaushal et al., 2000; Tang et al., 2001). To date, 20 members of the ADAMTS family have been identified in vertebrates (Alfandari et al., 1997; Kuno et al., 1997; Abbaszade et al., 1999; Hurskainen et al., 1999; Vazquez et al., 1999; Nath et al., 2000).

1.6.1: Structural and Functional Organisation of ADAMTS Subtypes

Members of the ADAMTS gene family are characterized by four structural and functional subunits: an amino terminal pro-domain, a catalytic domain, a disintegrin-like domain, and an ECM binding domain (which is composed of a central thrombospondin (TSP) type 1 motif, a spacer region and a variable number of TSP-like motifs) at the carboxy terminal end of the protein (Alfandari et al., 1997; Kuno et al., 1997; Abbaszade et al., 1999; Hurskainen et al., 1999; Vazquez et al., 1999; Nath et al., 2000; Kaushal et al.,
Overall, the predicted mature forms of the ADAMTS proteins are closely similar to one another. The prodomain of the distinct ADAMTS subtypes varies in length, but exhibits short stretches of homology that correspond to potentially important consensus sequences. In particular, the prodromes of the ADAMTS subtypes contain a Cys-switch motif, found in MMPs and SVMPs, that is involved in maintaining the enzyme in its latent form (Clark et al., 2000). A conserved furin activation site has also been identified in the prodromes of the ADAMTS. This putative cleavage site is located immediately prior to the amino terminal sequence of the mature protein suggesting that unlike MMPs, the prodromes of the ADAMTS subtypes are proteolytically cleaved by the endopeptidases in the Golgi apparatus and the proteins secreted in their active form. There is increasing experimental evidence to support this hypothesis (Tang et al., 1999; Cal et al., 2001; Fujikawa et al., 2001).

The catalytic domain of the ADAMTS contains consensus sequences also found in the proteolytic domains of SVMPs, ADAMs and/or MMPs (Rawlings et al., 1995). To date, the specific substrate(s) of many ADAMTS subtypes have not been identified. However, ADAMTS-4 and ADAMTS-8 have been shown to degrade aggrecan, a large chondroitin sulphate (Kuno et al., 1999; Rodriguez et al., 2000). ADAMTS-4 is also capable of degrading versican and the brain-specific ECM protein, BEHAB (Stocker et al., 1995; Tortorella et al., 2000). Procollagens-I and -II have been identified as substrates for ADAMTS-1, -2 and -3 (Nakamura et al., 2000; Tortorella et al., 2001). Although the
substrates of ADAMTS-10 and -12 have not been identified, the metalloprotease domains of these ADAMTS subtype have been shown to be proteolytically active using the α2-macroglobulin complex formation assay (Tang et al., 1999; Sandy et al., 2001).

*The disintegrin domain* is located immediately after the catalytic domain of the ADAMTS. This region shows limited homology to the disintegrin domains of ADAMs and SVMPs (Alfandari et al., 1997; Kuno et al., 1997; Abbaszade et al., 1999; Hurskainen et al., 1999; Tang et al., 1999; Vazquez et al., 1999; Nath et al., 2000). The disintegrin-like adhesion domain of this protein might interact with integrin-like receptors on cells to promote cell-matrix attachment or disrupt interaction between integrin receptors and the extracellular matrix (Miles et al., 2000).

*The ECM Binding Domain* contains an internal thrombospondin type 1 (TSP-1) motif that has two conserved regions, one of which is involved in binding sulphated glycosaminoglycan chains of heparin, heparan sulfate and chondroitin sulphate and the other in the binding of the thrombospondin receptor, CD36 (Prockop et al., 1998; Matthew et al., 2000). A spacer region of variable length separates this internal TSP motif from the TSP-1 repeats located at the carboxyl end of the protein. The spacer region exhibits the least sequence homology between the distinct members of the ADAMTS subfamily. The number of TSP-1 motifs at the carboxyl terminal of the protein is also highly variable among the ADAMTS subtypes (Alfandari et al., 1997; Kuno et al., 1997; Abbaszade et al., 1999; Hurskainen et al., 1999; Tang et al., 1999; Vazquez et al., 1999; Nath et al., 2000) (Fig. 1). The biological significance of these structural variations remains to be elucidated.
Figure 1. Diagram of ADAMTS subtype structures. Conserved structural motifs are shown. Despite motif conservation, amino acid sequences differ among ADAMTS subtypes.
The TSP-1 motifs and the spacer region are required for ADAMTS-ECM interactions. For example, the deletion of the central or the two carboxy terminal TSP motifs of ADAMTS-1 has been shown to inhibit its binding to ECM (Cal et al., 2001). The truncation of the spacer region also significantly reduced the ability of the mature protein to interact with the ECM. In contrast, deletion mutants corresponding to either the carboxy terminal TSP-1 or spacer region of ADAMTS-1 were capable of forming tight interactions with the ECM. These observations have led to the proposal that the spacer region of the ADAMTS may mediate specific interactions with distinct structural component(s) of the ECM.

Interactions between the ADAMTS subtypes and the ECM appear to be critical for their proteolytic activity. For example, truncated forms of ADAMTS-4 lacking the spacer region and/or TSP-1 motifs did not exhibit any protease activity in vitro (Rodriguez et al., 2000). Similarly, peptides corresponding to different regions of the TSP-1 motif and/or spacer region of ADAMTS-4 reduced the cleavage of its substrate, aggrecan, in a dose-dependent manner.

1.6.2: Tissue Distribution of the ADAMTS subtypes

ADAMTS subtypes have been detected in human adult and fetal tissues (Bornstein et al., 1994; Gantt et al., 1997; Magnetto et al., 1998; Zimmermann et al., 2001) (Tables 1 and 2, respectively). In addition, ADAMTS-1, -2, -3 and -8 mRNA levels have been shown to
<table>
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<tr>
<th>ADAMTS</th>
<th>Heart</th>
<th>Brain</th>
<th>TERM Placenta</th>
<th>Lung</th>
<th>Liver</th>
<th>Skeletal muscle</th>
<th>Kidney</th>
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Table 1. Distribution of the ADAMTS subtypes in adult human tissues. The relative mRNA expression levels of ADAMTS-1 to -12 are shown. +++ represents highest expression, and - represents no detectable mRNA (Reproduced from Bornstein et al., 1994; Gantt et al., 1997; Magnetto et al., 1998; Zimmermann et al., 2001).
Table 2. Distribution of the ADAMTS subtypes in human fetal tissues. The relative mRNA expression levels of ADAMTS-9 and -12 are shown. +++ represents highest expression, and – represents no detectable mRNA (Reproduced from Bornstein et al., 1994; Gantt et al., 1997; Magnetto et al., 1998; Zimmermann et al., 2001).

<table>
<thead>
<tr>
<th>ADAMTS</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Lung</th>
<th>Skeletal muscle</th>
<th>Spleen</th>
<th>Thymus</th>
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Table 3. ADAMTS mRNA levels in the developing mouse fetus. The relative mRNA expression levels of ADAMTS-1, -2, -3 and -8 are shown. ++ represents highest expression, and – represents no detectable mRNA (Reproduced from Fernandes et al., 2001).

<table>
<thead>
<tr>
<th>ADAMTS</th>
<th>Days of gestation</th>
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<tr>
<td></td>
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<td>8</td>
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be highly regulated during the early stages of embryonic development in the mouse (Fernandes et al., 2001) (Table 3).

### 1.6.3: The Cell Biology of the ADAMTS

To date, the majority of the distinct ADAMTS subtypes have only been characterized at the structural level. Consequently, the biological functions of many of these novel proteases under normal and pathological conditions has not been determined. However, recent gene knockout studies have highlighted the important biological roles that members of the ADAMTS gene family play in embryonic development and tissue morphogenesis (Shindo et al., 2000; Li et al., 2001).

ADAMTS-1 is a new member protein of the ADAM family. It was identified by differential display analysis as a gene highly expressed in the murine colon 26 cachexigenic tumor (Kuno et al., 1997). ADAMTS-1 gene knockout mice exhibit growth retardation and aberrant development of the kidneys, adrenal glands and urogenital tract (Shindo et al., 2000). Abnormalities in the ovaries and uterine tissues of female mice null mutant for ADAMTS-1 were also observed. In particular, the ovaries of these mice contained fewer mature follicles, supporting previous observations suggesting a role for ADAMTS-1 in folliculogenesis (Espey et al., 2000). The large cysts present in the endometrial cell layers of these mice are also likely to contribute to the reduced number of implantation sites observed at day 10 of pregnancy.
Site directed mutagenesis of ADAMTS-1 revealed that the thrombospondin type I motifs, together with the carboxyl-terminal spacing region, are responsible for anchoring to sulfated glycosaminoglycans such as heparan sulfate of the extracellular matrix (Kuno et al., 1998). Because ADAMTS-1 has a potential zinc-binding motif in the metalloproteinase domain, recent studies using a proteinase trapping mechanism with α2-macroglobulin as a substrate demonstrated that the metalloproteinase domain of ADAMTS-1 is a secreted active ADAM protease that is closely associated with the extracellular matrix through the complex formation with α2-macroglobulin (Kuno et al., 1999). A point mutation within the Zn-binding motif abolished complex formation. Thus, ADAMTS-1 is involved in proteolytic modification of cell-surface proteins and extracellular matrices. Electron microscopy showing the accumulation of collagen fibers suggest that processing of collagen and related matrix substances may be impaired in ADAMTS-1<sup>−/−</sup> mice (Colige et al., 1997). In addition, ADAMTS-1 degrades to different extents the cartilage proteoglycans aggrecan and lectican, or aggrecan-like proteins such as brevican and versican (Jean et al., 2004). However, it is difficult to assign the molecular substrate(s) of ADAMTS-1 because the characteristics of histological changes seem to be different among organs. The discovery of physiologically relevant substrate(s) of ADAMTS-1 would be likely to pave the way for further understanding of growth, fertility, and organ morphogenesis (Shindo et al., 2000).

ADAMTS-1 is synthesized as a zymogen, which requires activation via proteolytic removal of a pro-domain. Furin, which is concentrated in the trans-Golgi network (TNG) has been demonstrated to be the convertase that is most efficient at cleaving
proADAMTS-1 (Jean et al., 2004). A previous study also showed that removal of the pro-domain from the ADAMTS-1 precursor is impaired in the furin-deficient cell line, LoVo, and the processing ability of the cells is restored by co-expression of furin cDNA (Kuno et al., 1998). The cysteine switch model predicts that metalloproteinases are kept latent by the interaction of a conserved Cys residue of the prodomain and a zinc atom in the catalytic domain that blocks the active site. Disruption of this interaction leads to removal of the prodomain and activation of the enzyme (Van et al., 1990).

Mice null mutant for the ADAMTS-2 gene did not exhibit any abnormal phenotype at birth. However, as these mice matured, their skin became fragile (Li et al., 2001). At the structural level, the skin of these mice mimicked the defects described for the connective tissue disorder known as dermatosparaxis in animals and Ehlers-Danlos syndrome in humans (Colige et al., 1997; Giunta et al., 1999). Furthermore, male mice null mutant for ADAMTS-2 were infertile. Infertility in these mice was attributed to a reduced number of sperm in the seminiferous tubules suggesting that ADAMTS-2 plays a key role in spermatogenesis.

Aberrant expression of ADAMTS subtypes has also been associated with the pathogenesis of disease. For example, ADAMTS-12 mRNA levels were found to be significantly higher in colorectal, renal and pancreatic carcinomas than in matched normal tissues (Cal et al., 2001). Similarly, increased ADAMTS-4 expression and proteolytic activity have been detected in gliomas of the CNS (Bayliss et al., 2001). ADAMTS-4 and -5 have also been shown to play key roles in the degradation of cartilage during the
progression of arthritic diseases (Tortorella et al., 2000). Finally, it has been proposed that ADAMTS-4 is involved in the degradation of the ECM in the brains of patients diagnosed with Alzheimer’s disease (Satoh et al., 2000).

Collectively, these observations suggest that the ADAMTS play key role(s) in tissue morphogenesis during embryonic development and maintain the integrity of tissues in the adult.

1.6.4: Regulation of ADAMTS Expression

The factors capable of regulating ADAMTS expression remain poorly characterized. TGF-1β, but not TGF-α, IL-α, IL-1β, αFGF or EGF increased ADAMTS-12 mRNA levels in human fetal fibroblasts (Cal et al., 2001). The inflammatory cytokine, interleukin-1, could induce the mouse ADAMTS-1 transcript in colon 26 cells. Lipopolysaccharides increase ADAMTS-1 mRNA levels in renal and cardiac tissues of adult mice whereas P4 appears to be a key regulator of this ADAMTS subtype in the rat ovary (Kuno et al., 1997; Robker et al., 2000).

1.6.5: Regulation of ADAMTS Activity

*Proteolytic Modification:* Recent studies indicate that ADAMTS-1, -4 and -12 undergo a second proteolytic cleavage, resulting in the formation of an amino terminal fragment containing the metalloproteinase, disintegrin-like and the central TSP-1 domains of the mature proteins (Kuno et al., 1999; Tang et al., 1999; Cal et al., 2001). The truncated
forms of these proteins have modified adhesion properties and therefore, are likely to have different biological functions. For example, the amino terminal fragment of ADAMTS-1 binds to the ECM with a lower affinity and is less effective at regulating the proliferation of endothelial cells than the mature form (Cal et al., 2001). Thus, the removal of the TSP motifs may serve as endogenous mechanism that regulates the catalytic activity of this ADAMTS subtype. It has also been proposed that the carboxy terminal fragments of ADAMTS-4 and -12, which are also capable of binding to the ECM, may function as competitive inhibitors of the enzymatic activity of the mature proteins (Tang et al., 1999; Kuno et al., 1999).

_Endogenous Regulatory Factors:_ TIMP-3, but not TIMPs-1, -2, or -4, has been shown to be a potent inhibitor of recombinant ADAMTS-4 and ADAMTS-5 _in vitro_ (Kashiwagi et al., 2001). To date, the molecular mechanism(s) by which TIMP-3 inhibit these two ADAMTS subtypes have not been characterized. However, the unique structural properties (see Section 1.2.3-B) of TIMP-3 that allows it to bind to the ECM (Yu et al., 2000) may be important in the localized regulation of ADAMTS-4 and ADAMTS-5 proteolytic activity.

Papilin is an ECM glycoprotein, identified in _Drosophila, C. elegans_ and mammals, which inhibits the proteolytic activity of ADAMTS-1 _in vitro_. Papilin shares a set of protein domains (the papilin cassette) with the ADAMTS (Kramerova et al., 2000). In particular, the papilin cassette contains a complete TSP type 1 repeat, a spacer region, and six incomplete TSP type 1 domains. In binding assays, papilin was capable of interacting with ADAMTS-1 and the enzyme-substrate complex, but did not compete with procollagen.
for the catalytic site of this proteinase. In view of these observations, it has been proposed that papilin regulates the proteolytic activity of ADAMTS subtype(s) by binding to its spacer region and/or TSP-1 motifs, thereby preventing interaction(s) with the ECM.

Alterations in the expression levels of papilin have a profound effect on the development of the *Drosophila* embryo (Kramerova et al., 2000). For example, over-expression of papilin resulted in the aberrant development of the Malphigian tubules, muscle, trachea, CNS, and death of these embryos. Similarly, inhibition of papilin expression in adult *C. elegans* markedly reduced brood sizes and often caused the death of the parental animal (Kramerova et al., 2000). The few offspring which survived were infertile. The mechanisms by which papilin modulates these morphogenetic events have yet to be elucidated. Furthermore, the biological role(s) of papilin in mammalian tissue morphogenesis has not been determined.

### 1.6: Hypothesis

In view of these observations, we have hypothesized that member(s) of the ADAMTS gene family mediate, at least in part, the endometrial ECM remodeling events during early pregnancy. This hypothesis is supported by aberrant endometrial development of mice null-mutant for the ADAMTS-1 gene, which in turn, likely contributes to the subfertility of these animals. Since the structures of ADAMTS subtypes are highly conserved, it is likely that other ADAMTS family members may play similar or related roles. In these studies, we have undertaken a comprehensive survey of the ADAMTS subtypes present in the human
endometrial decidua tissue during early pregnancy. In addition, we have examined the ability of IL-1β and TGF-β1, two cytokines that play key regulatory roles during early pregnancy, to regulate ADAMTS-1 mRNA and protein expression levels in primary cultures of stromal cells isolated from first trimester human decidual tissues.

1.7: Specific Aims

Specific Aim 1: To perform a comprehensive survey of the ADAMTS subtypes present in first trimester human decidual tissues.

A comprehensive survey of the ADAMTS subtypes present in first trimester human decidual tissues will be carried out using a reverse transcription-polymerase chain reaction (RT-PCR) strategy. Total RNA extracts prepared from first trimester tissues will be prepared and used to synthesize cDNA. This will be utilized for RT-PCR with primers specific for ADAMTS-1 through 12. PCR reaction mixtures containing non-reverse transcribed RNA or water will be used as negative controls and placenta cDNA will be used as a positive control. The PCR cycles will be repeated 20-40 times to determine a linear relationship between the yield of PCR products and number of cycles.

Specific Aim 2: To determine whether IL-1 and/or TGF-β1 are capable of regulating ADAMTS-1 mRNA and protein expression levels in primary cultures of human decidual stromal cells in a dose- and time-dependent manner.
The regulated expression of the ADAMTS-1 is believed to play key roles in the highly regulated series of remodeling events that occur in the endometrium in preparation for pregnancy. To gain a better understanding of the role(s) of IL-1β and TGF-β1 in this developmental process, we will examine the abilities of IL-1β and TGF-β1 to regulate ADAMTS-1 mRNA and protein expression levels in a dose- and time-dependent manner in primary cultures of stromal cells isolated from first trimester decidual tissue using QC-PCR and Western blotting respectively.
PART II. GENERAL MATERIALS AND METHODS

2.1: Tissues

Tissue samples of first trimester decidua parietalis were obtained from women undergoing elective termination of pregnancy (gestation ages ranging from 6-12 weeks). The use of these tissues was approved by the Committee for Ethical Review of Research on the Use of Human Subjects, University of British Columbia. All patients provided informed written consent.

2.1.1: Cell Isolation and Culture

Stromal cells were isolated from the decidual tissue samples by enzymatic digestion and mechanical dissociation using a protocol modified from that reported by Shiokawa et al. (1996). Briefly, the decidual tissue samples were minced and subjected to 0.1% collagenase (type IV) (Sigma Chemical Co, St Louis, MO) and 0.1% hyaluronidase (type I-S) (Sigma Chemical Co, St Louis, MO) digestion in a shaking water bath at 37 °C for 60 minutes. The cell digest was then passed through a nylon sieve (38 μm) (Becton Dickinson and Co, Franklin Lakes, NJ). The isolated glands and any undigested tissue fragments were retained on the sieve, and the eluate containing the stromal cells was collected in a 50 ml tube. The stromal cells were then pelleted by centrifugation at $800 \times g$ for 10 minutes at room temperature. The cell pellet was washed once with DMEM containing 10% fetal bovine serum (FBS) before being resuspended and plated in DMEM containing 25mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and
supplemented with 10% FBS, 17β-estradiol (10 nM; Sigma Chemical Co) and progesterone (1 μM, Sigma Chemical Co). The culture medium was replaced 30 minutes after plating to reduce epithelial cell contamination. This protocol was previously demonstrated to result in pure decidual stromal cells through the absence of immunocytochemical staining for the markers vimentin (fibroblast), cytokeratin (epithelial), muscle actin (muscle cells), and factor VIII (endothelial) (Chou et al., 2002).

2.2: Experimental Culture Conditions

Decidual stromal cells (passage 2) were plated in 35 mm² tissue culture dishes (Becton Dickinson and Co, Franklin Lakes, NJ) at a density of 1 x 10⁵ cells/dish and grown to 80% confluence. The cells were starved for 24 hours before cultured in the presence of increasing concentrations of IL-1β (0, 1, 10, 100 or 1000 IU/ml), or TGF-β1 (0, 0.1, 1, 5, or 10 ng/ml) for 24 hours or a fixed concentration of IL-1β (100 IU/ml) or TGF-β1 (5 ng/ml) for 0, 6, 12, 24 or 48 hours. The concentrations of cytokines used in these studies are based upon previous studies (Huang et al., 1998).

To block the regulatory effects of IL-1β and TGF-β1 on ADAMTS-1 mRNA and protein expression levels in these primary cell cultures, decidual stromal cells were cultured in the presence of either IL-1β (100 IU) in combination with a monoclonal antibody directed against human IL-1β (1 ug/ml or 2 ug/ml; Sigma Aldrich) for 24 hours, or with TGF-β1 (5 ng/ml) alone, or in combination with a monoclonal antibody directed against human TGF-β1 (10 ug/ml; Sigma Aldrich, St Louis, MO).
All of the primary cultures of decidual cells were harvested for either total RNA or protein extraction.

2.3: RNA Preparation and Synthesis of First Strand cDNA

Total RNA was prepared from decidual tissues cell cultures using the Tri-Reagent (Bio/Can, Mississauga, Canada) using a protocol recommended by the manufacturer. The concentration of total RNA present in each of the extracts was quantified by optical densitometry (260/280nm) using a DU-64 UV-spectrophotometer (Beckman Coulter).

RNA prepared from the decidual stromal cells was reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Oakville, Canada). An aliquot (1μg) of the total RNA dissolved in DNase/ RNase-free water (8 μl in total) was heated at 65 ºC for 10 minutes and cooled on ice for 5 minutes. Dithiothreitol (DTT) (1 μl), oligo-dT (1 μl) and bulk mixture (dATP, dCTP, dGTP, dTTP) (5 μl) was added to the sample, and the mixture was incubated at 37 ºC for 1 hour. After incubation, the sample was boiled for 10 minutes to inactivate reverse transcriptase and subsequently stored at -20 ºC until use.

Several controls were included to determine the accuracy of the PCR. Firstly, PCR amplification was performed in both the absence of cDNA and reverse transcription reaction to examine the cross-contamination of samples. Secondly, the integrity of the RNA samples was confirmed by gel electrophoresis. In addition, the parallel PCR
amplification of GAPDH was performed to determine the quality of the isolated RNA and rule out the possibility of RNA degradation. Lastly, since all primer pairs spanned at least one intron, the size of the predicted PCR products ruled out the presence of contaminating genomic DNA in the RNA sample.

2.3.1: Design of Oligonucleotide Primers

Nucleotide sequences specific for human ADAMTS-1 through -12 cDNAs were identified in the Genebank database using the BLAST (Basic Local Alignment Search Tool) computer program (NCBI, Bethesda, MD). Forward and reverse oligonucleotide primers corresponding to these DNA sequences and primers specific for GAPDH, which served as an internal control for these studies, were synthesized at the NAPS Unit, University of British Columbia. In addition, a competitive forward primer for ADAMTS-1 was designed through the incorporation of an additional 10 base pairs into 3' end of original forward primer (Fig. 2). The specific nucleotide sequences of these primers, PCR product sizes and corresponding position in cDNA are listed in Table 4.

The specific nucleotide sequences of the primers, the optimized PCR conditions for each of these primer sets, and the expected sizes of the PCR products for the semi-quantitative analysis of ADAMTS subtypes mRNA levels are listed in Table 5.
Figure 2. Representative schematic diagrams summarizing construction of a competitive PCR primer for ADAMTS-1. An internal standard fragment (321 bp) was constructed by deletion of a 299 bp fragment from the target cDNA (620 bp).
<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers 5'-3'</th>
<th>Size(bp)</th>
<th>Position cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS-1</td>
<td>Forward (5' end) CGAGTGTGCAAGGAAGTGGA</td>
<td>620</td>
<td>2912-2931</td>
</tr>
<tr>
<td></td>
<td>Reverse (3'end) ATCATAGTACCCCCCAACCCT</td>
<td></td>
<td>3530-3511</td>
</tr>
<tr>
<td>ADAMTS-1</td>
<td>Competitive(5' end) CGAGTGTGCAAGGAAGTGAGCTT</td>
<td>321</td>
<td>2912-2931, 3231-3240</td>
</tr>
<tr>
<td></td>
<td>Reverse (3'end) ATCATAGTACCCCCCAACCCT</td>
<td></td>
<td>3530-3511</td>
</tr>
</tbody>
</table>

Table 4. Primer sequences, and their corresponding position in cDNA for the QC-PCR analysis of ADAMTS-1 mRNA levels in decidual stromal cells. A forward and a reverse primers produce a 620 bp target cDNA and a competitive forward primer and a reverse primer produce a 321 bp internal standard cDNA.
Table 5. Primer sequences and PCR conditions for the semi-quantitative analysis of ADAMTS subtype mRNA levels.

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Estimated PCR Product Size</th>
<th>PCR Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS-1</td>
<td>399bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 65°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
</tr>
<tr>
<td>Forward: 5’-CGAGTGTGCAAAGGAAGTGA-3’&lt;br&gt;Reverse: 5’- CTACCCCATATAATCCCACCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAMTS-2</td>
<td>310bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 65°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
</tr>
<tr>
<td>Forward: 5’-CCTATGACTGGCTGCTGGAT-3’&lt;br&gt;Reverse: 5’-CTCCCCAAAGGTGCTGGGATAA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAMTS-3</td>
<td>444bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 65°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
</tr>
<tr>
<td>Forward: 5’-CTGTGGTGAGGTTTCCAGT-3’&lt;br&gt;Reverse: 5’-CTGACCGACTCAGGTTTTTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAMTS-4</td>
<td>349bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 60°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
</tr>
<tr>
<td>Forward: 5’-AATCCAGGGTGTTGTTGATA-3’&lt;br&gt;Reverse: 5’-TACTCAGGAGGCTGAGGCAT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAMTS-5</td>
<td>444bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 65°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
</tr>
<tr>
<td>Forward: 5’-GGCCATGGTAACTGTTTGTCT-3’&lt;br&gt;Reverse: 5’-CCTCTTTCCCTGTGCAATGAC-3’</td>
<td></td>
<td></td>
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<td>ADAMTS-6</td>
<td>340bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 60°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
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<td>Forward: 5’-TGACAGTCCAGCACCTTCAG-3’&lt;br&gt;Reverse: 5’-CTACGTGCTTGTGCATTCTCCA-3’</td>
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<td></td>
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<tr>
<td>ADAMTS-7</td>
<td>389bp</td>
<td>Denaturing: 94°C 30s&lt;br&gt;Annealing: 55°C 30s&lt;br&gt;Extension: 72°C 60s&lt;br&gt;35 cycles</td>
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<td>Forward: 5’-CCATGTGGTGTAACAAGCGTC-3’&lt;br&gt;Reverse: 5’-GGTCCTCTCCTCCTCATCTTCC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ADAMTS-8</td>
<td>ADAMTS-9</td>
</tr>
<tr>
<td>---</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td></td>
<td>Forward: 5'- AAGAAGAGGAGGCCAGAAGGC-3'</td>
<td>Reverse: 5'- TCTGTCTGGGTGAGCAGGATG-3'</td>
</tr>
<tr>
<td></td>
<td>Forward: 5'- CCCAGCCTGGACACATTACT-3'</td>
<td>Reverse: 5'- CATTAGCCTGGACTCCCACA-3'</td>
</tr>
<tr>
<td></td>
<td>Forward: 5'- CAATGTCCTCATTGACGCTG-3'</td>
<td>Reverse: 5'- CTGGGAAGCACCCTAACCAT-3'</td>
</tr>
<tr>
<td></td>
<td>Forward: 5'- GTGCAGCGAGGAGTACATCA-3'</td>
<td>Reverse: 5'- GCGTTTTCTTTCTCCAGTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Forward: 5'- ATGTTTCGTCATGGGTGTAACCA-3'</td>
<td>Reverse: 5'- TGGCAGGTTTTTCTAGACGGCAG-3'</td>
</tr>
</tbody>
</table>
To confirm the specificity of the primers, the amplified PCR products were sub-cloned into the PCR II vector (Invitrogen, Carlsbad, CA) by blunt-end ligation and subjected to nucleotide sequence analysis using an automated DNA sequence analyzer (Applied Biosystems, Foster City, CA) employing Taq DiDeoxy reagents (Performed by DNA Sequencing Core Facility, CMMT, University of British Columbia).

2.32: Semiquantitative RT-PCR

PCR was performed using the primer sets specific for ADAMTS-1 through -12, and template cDNA generated from the total RNA extracts prepared from the first trimester decidua or stromal cells isolated from these tissues. PCR reaction mixtures containing non-reverse transcribed RNA or water will be used as negative controls and placenta cDNA as positive control. The PCR cycles were carried out 20-40 times to determine a linear relationship between the yield of PCR products and cycle number.

2.4: Quantitative Competitive-Polymerase Chain Reaction (QC-PCR)

The QC-PCR strategy employed in these studies was based upon the competitive co-amplification of known amounts of a competitive ADAMTS-1 PCR product added to the aliquots of first strand cDNA prepared from the primary cultures of decidual stromal cells cultured in the presence or absence of IL-1β or TGF-β1.

Semi-quantitative PCR was also performed using template cDNA generated from the
total RNA extracts prepared from cultures of untreated decidual stromal cells and the primers specific for ADAMTS-1. The PCR conditions were as follows: 1 min at 94 °C, 1 min at 58.5 °C and 1.5 mins at 72 °C followed by a final extension at 72 °C for 15 min. The cycles were repeated 28 times.

The 620 bp ADAMTS-1 PCR product was resolved using gel (2%) electrophoresis and visualized by ethidium bromide staining. An aliquot of the eluted 620 bp fragment was then subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequence analysis to confirm the specificity of the primers.

Similarly, PCR amplification template cDNA generated from the decidual stromal cell cultures using a combination of the reverse primer specific for ADAMTS-1 and the corresponding competitive primer yielded a truncated cDNA fragment of the expected size of 321 bp. This PCR product was also subcloned into the PCR II vector and subjected to DNA sequence analysis to confirm the specificity of these primer sets.

To determine the ideal amounts of each template to be added to the reaction mixtures, PCR was performed using fixed amounts of the target ADAMTS-1 cDNA (1µl) and decreasing concentrations of the corresponding internal standard cDNA obtained by serial dilution of the first strand cDNA preparations (1- 0.003907 pg/µl for ADAMTS-1). One microliter each of the mutant and native cDNA mix were added to 23µl PCR-Mastermix containing 1.5 mmol/liter MgCl₂ solution, 10X PCR buffer minus Mg²⁺, 0.2 mmol/liter of each deoxy-nucleoside 5’-triphosphate, 2.5 U Taq polymerase (all from Life Technologies,
Inc.), and their corresponding paired primers (2 μmol/liter) to give a total volume of 25μl. The PCR conditions and number of cycles were performed for the ADAMTS-1 primer sets as described above.

An aliquot (10μl) of the PCR reaction mixture containing the two distinct ADAMTS-1 PCR products was subjected to electrophoresis in a 1% agarose gel and visualised by ethidium bromide staining (Refer to Figure 4 at Part III. Results). The intensity of the ethidium bromide staining of the PCR products was analysed using UV densitometry (Biometra, Whiteman Co., Gottigen, German). Volume counts (mm$^2$) of the PCR products were then determined using the Scion Image computer software (Scion Image Co, Frederick, Maryland). The absorbance values obtained for each of the target and corresponding internal standard were plotted against the amount of internal standard added to the initial reaction mixtures, with the point of interception on these line graphs being taken as the optimal amount of internal standard to be used in the QC-PCR analysis. The intensity of the ethidium bromide staining of an aliquot (10 μl) of a 100-bp DNA ladder (Life Technologies Inc, Mississauga, Ontario, Canada) served as an internal standard.

PCR was also used to co-amplify the optimized amounts of the ADAMTS-1 internal standard cDNA and increasing amounts of the corresponding target cDNA (1-0.003907 pg/μl). The ratios of the intensity of the ethidium bromide staining of the resultant target:internal standard PCR product generated in each tube were logarithmically transformed and plotted against the log amount of target cDNA initially added to the PCR reaction (Refer to Figure 5 at Part III. Results). This standard curve was highly reproducible and linear. The
values obtained from this regression curve \( y = b + mx \) was subsequently used to quantify the levels of the ADAMTS-1 mRNA transcript present in the decidual stromal cell cultures.

Based upon these initial observations, an aliquot containing 0.0625 pg of the competitive ADAMTS-1 cDNA was subsequently added to each aliquot of the first strand cDNA generated from our human decidual stromal cell cultures and subjected to QC-PCR. QC-PCR was performed using ADAMTS-1 primer sets and the PCR conditions described above, with 1 μl of the first strand cDNA synthesized from each of the decidual stromal cell cultures. The ratios of native:competitive ADAMTS-1 cDNAs were determined as described above, logarithmically transformed and compared with the values obtained from the standard curve.

2.5: Western Blot Analysis

Decidual stromal cell cultures were treated with IL-1β and TGF-β1 in the presence or absence of their neutralizing inhibitor for 2 hours were washed three times in cold 1% PBS and incubated in 100 μl of cell lysis buffer (25 mM Tris HCl, pH 7.6 containing 2% Nonidet P-40, 50 mM NaCl, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 50 μM leupeptin) at 4 °C for 30 minutes on a rocking platform. The cell lysates were clarified by centrifugation in an Eppendorf microcentrifuge for 20 minutes. The concentration of protein in the supernatants was determined using the BCA kit (Pierce Chemicals, Rockford, IL). The supernatants (20 μg protein) were mixed with 4X Laemmli sample buffer and boiled for 5 min. Western
blots were prepared and immunoblotted as previously described (MacCalman et al., 1996). Prepared samples were electrophoresed through a 12% SDS-polyacrylamide gel and the separated proteins were then electrophoretically transferred onto nitrocellulose membrane (Hybond-C, Amersham-Pharmacia Biotech, Morgan, Canada). Membranes were incubated in blocking buffer (TTBS; 25mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20) (containing 5% skim milk) for 90 minutes at room temperature; then a polyclonal rabbit antibody directed against the carboxyl terminus of human ADAMTS-1 (Biodesign International, Saco, ME) (1:1000) was added to the blocking buffer (1X TTBS with 5% skim milk) (10ml/membrane), and blots were incubated overnight at 4°C. The blots were rinsed and washed three times (10 min each) in 1X TTBS without skim milk and incubated with Horseradish Peroxidase (HRP)-conjugated anti-rabbit Ig (Amersham; 1:3000 in1X TTBS with 5% skim milk) (10ml/membrane) for 1 hour at room temperature on a rocking platform.

The Amersham ECL system was used to detect antibody bound to antigen. Membranes were visualized by exposure to Kodak X-Omat film Eastman Kodak Co., Rochester, NY) at -70°C. The resultant autoradiograms were then scanned using a laser densitometer.

2.6: Statistical Analysis

Results from the densitometric quantification of DNA or protein bands respectively detected on ethidium bromide stained gels or autoradiograms obtained from Western
blotting were subjected to statistical analysis using GraphPad Prism 4 computer software (San Diego, CA, USA). Statistical differences were assessed using analysis of variance (ANOVA). Differences were considered significant at \( p \leq 0.05 \). Significant differences between the means were determined using Tukey's test. The results are presented as the mean relative absorbance (± S.E.M), in arbitrary units, obtained from 3 independent experiments.
PART III. RESULTS

3.1: Multiple ADAMTS subtypes are present in first trimester decidual tissues

To date, there is nothing known of the expression of any ADAMTS family members in decidual tissue. To establish which ADAMTS subtypes are expressed in early stage deciduas, primers specific for selected ADAMTS members (ADAMTS-1 through -12) were designed and utilized in a RT-PCR strategy (refer to Materials and Methods, sections 2.3.1 and 2.3.2). This revealed that mRNA transcripts encoding ADAMTS-1, -2, -3, -4, -5 (-11), -6, -7, -8, -9, -10 and -12 family members were present in first trimester decidual tissue (Fig. 3).

3.2: Dose-dependent effects of IL-1β and TGF-β1 on ADAMTS-1 mRNA and protein levels in human decidual stromal cells

If ADAMTS proteins play important roles in the extensive tissue remodeling that occurs during embryo implantation and through pregnancy, it is likely that their expression is dynamically and tightly regulated throughout these processes. As the gene-targeted disruption of ADAMTS-1 in mouse was shown to reduce pregnancy rates (Shindo et al., 2000), I focused on investigating the regulated expression of this ADAMTS subtype in human decidua, using a model system of cultured decidual stromal cells. IL-1β and TGF-β1 were chosen as modulators because these cytokines are highly expressed during the first trimester of pregnancy (Lala et al., 1990; Graham et al., 1991; Simon et al., 1994). In addition, these cytokines are able to regulate the expression of
Figure 3. Characterization of the ADAMTS mRNA transcripts present in human decidual tissue. RT-PCR was performed using template cDNA, synthesized from total RNA extracted from first trimester decidual tissue, and primers specific for ADAMTS-1 through-12. Total RNA and DNase/RNase free water (H20) served as negative controls for the present studies. The sizes of the distinct PCR products are shown below each photomicrograph. bp, base pairs.
other proteases such as matrix metalloproteinase (MMP) and urokinase plasminogen activator (uPA) at the maternal-fetal interface (Robert et al., 1988; Lala et al., 1990; Graham et al., 1997; Huang et al., 1998).

In order to quantitate ADAMTS-1 mRNA in the decidual stromal cells, (QC)-PCR was performed using a fixed amount of cDNA (1 μl) from an untreated decidual stromal cell culture, co-amplified with a serial diluted competitive cDNA (refer to Materials and Methods, section 2.4). This co-amplification determined that 0.0625 pg/μl (internal standard) ADAMTS-1 mRNA transcript was present in these cells (Fig. 4). A standard curve was then generated by co-amplifying a fixed concentration of competitive cDNA (0.0625 pg/μl) and a decreasing concentration of target cDNA (Fig. 5). This standard curve was highly reproducitive and linear. The values obtained from a regression curve (y = b+mx) was subsequently used to quantify the levels of the ADAMTS-1 mRNA transcript present in the decidual stromal cultures.

Similarly, a protein species of approximately 110 kDa corresponding to the proform of ADAMTS-1 was detected in the primary decidual stromal cell cultures by Western blotting of cell lysates with ADAMTS-1 antibody (Figs. 6-9).

Increasing concentrations of IL-1β increased the levels of the ADAMTS-1 mRNA transcript present in these cells in a dose-dependent manner (Fig. 6A). However, significant increases in ADAMTS-1 mRNA levels were only observed in decidual stromal cells treated with the higher concentrations of IL-1β (100 and 1000 IU/ml) used in these studies.
Figure 4. Quantification of ADAMTS-1 mRNA in human endometrial decidual stromal cells. Photomicrograph representing an ethidium bromide stained gel containing PCR products generated using a fixed amount of target cDNA (1 μl) and serial dilutions of concentrations of competitive cDNA (1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 pg/μl). (upper panel). The two lines in the range of 0.125-0.063 pg/μl internal standard cDNA, indicating that approximately 0.0625 pg ADAMTS-1 cDNA could be detected by RT of 1 μg total RNA (lower panel).
Figure 5. Generation of a standard curve for ADAMTS-1 mRNA in human endometrial decidual stromal cells. Photomicrograph of an ethidium bromide stained gel containing PCR products generated using a decreasing amount of target cDNA co-amplified with 0.0625 pg/μl competitive cDNA using the primer set specific for ADAMTS-1. The resultant sizes of the PCR products were compared to a 100-bp ladder (marked M, on the left side of the photomicrograph). The densities of the ethidium bromide staining of the ratio of target and competitive cDNA produced from these reaction mixtures were determined using UV densitometry (upper panel). The log amount of target cDNA (pg) is shown in the graph.
Figure 6. Expression of ADAMTS-1 in decidual cells cultured in the presence of increasing concentrations of IL-1β. A, Representative photomicrograph of an ethidium-stained gel containing QC-PCR products generated by using template cDNA produced from endometrial decidual stromal cells cultured in the presence of increasing concentrations of IL-1β. QC-PCR analysis of ADAMTS-1 mRNA levels in decidual cells cultured in the presence of 0, 1, 10, 100, 1000 IU of IL-1β. The sizes of the resultant target (620 bp) and internal standard (321 bp) co-amplification PCR products relative to a 100-bp ladder (M) are marked on the right of the photomicrograph. The results derived from this analysis as well as the other two studies are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. untreated control). B, Western blot analysis of ADAMTS-1 expression in protein extracts (20μg) prepared from decidual stromal cells cultured in the presence of 0, 1, 10, 100, 1000 IU of IL-1β for 24 hours (lanes 1-5 respectively). The results derived from this analysis and two other studies are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. untreated control).
IL-1β at 100 IU/ml induced approximately a 2.1-fold increase in ADAMTS-1 mRNA, while 1000 IU/ml induced an approximate 3-fold increase.

In agreement with the results obtained using QC-PCR, IL-1β increased ADAMTS-1 protein expression levels in decidual stromal cells cultures in a dose-dependent manner (Fig. 6B). In excellent correspondence with the fold-increases in mRNA, ADAMTS-1 protein expression was increased 2.4-fold by 100 IU/ml of IL-1β, and 2.8-fold by 100 IU/ml of IL-1β.

In contrast, TGF-β1 decreased ADAMTS-1 mRNA levels in a dose-dependent manner. A significant decrease in ADAMTS-1 mRNA was observed only in primary cultures of decidual stromal cells treated with the higher concentrations of TGF-β1 (5 ng and 10 ng/ml) used in these studies (Fig. 7A). TGF-β1 at 5 ng/ml induced a 2.6-fold decrease in ADAMTS-1 mRNA, while treatment with 10 ng/ml TGF-β1 resulted in a 5-fold decrease in ADAMTS-1 mRNA expression. TGF-β1 treatment also reduced ADAMTS-1 protein expression levels in primary cultures of decidual stromal cells in a dose-dependent manner (Fig. 7B). In accord with the fold-decreases in mRNA, ADAMTS-1 protein expression was decreased 2.8-fold and 5-fold by 5 and 10 ng/ml of TGF-β1, respectively.
Figure 7. Expression of ADAMTS-1 in decidual cells cultured in the presence of increasing concentrations of TGF-β1. A, Representative photomicrograph of an ethidium-stained gel containing QC-PCR products generated by using template cDNA produced from endometrial decidual stromal cells cultured in the presence of increasing concentrations of TGF-β1. QC-PCR analysis of ADAMTS-1 mRNA levels in decidual cells cultured in the presence of 0, 0.1, 1, 5, or 10 ng of TGF-β1 for 24 hours (lanes 1-5 respectively). The sizes of the resultant target (620 bp) and internal standard (321 bp) co-amplification PCR products relative to a 100-bp ladder (M) are marked on the right of the photomicrograph. The data derived from this analysis as well as those from 2 other studies were standardized to the mean obtained from the untreated control and are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. untreated control). B, Western blot analysis of ADAMTS-1 expression in protein extracts (20μg) prepared from decidual stromal cell cultures. Decidual cells were cultured in the presence of 0, 0.1, 1, 5, or 10 ng of TGF-β1 for 24 hours (lanes 1-5 respectively). The data derived from this analysis and two other studies are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. untreated control).
3.3: Time-dependent effects of IL-1β and TGF-β1 on ADAMTS-1 mRNA and protein levels in human decidual stromal cells

IL-β1 (100 IU/ml) caused a significant increase in ADAMTS-1 mRNA transcription levels after 24 hours of culture with maximum levels being detected in cells cultured in the presence of this cytokine for 48 hours (Fig. 8A). Treatment with IL-β1 (100 IU/ml) for 24 hours induced approximately a 2.6-fold decrease in ADAMTS-1 mRNA, while the same treatment for 48 hours induced approximately a 2.9-fold increase. In excellent correspondence with the fold-increases in mRNA, ADAMTS-1 protein expression in decidual stromal cell cultures was increased 2.4-fold by treatment with IL-β1 (100 IU/ml) for 24 hours, and increased 2.5-fold upon treatment for 48 hours (Fig. 8B).

In contrast, a significant decrease in ADAMTS-1 mRNA transcription levels was detected in decidual stromal cells cultured in the presence of TGF-β1 (5 ng/ml) for 24 and 48 hours (Fig. 9A). Treatment with TGF-β1 (5 ng/ml) for 24 hours induced approximately a 2.4-fold decrease in ADAMTS-1 mRNA, while the same treatment for 48 hours induced approximately a 5.4-fold in these primary cell cultures. In agreement with the results obtained using QC-PCR, ADAMTS-1 protein expression level decreased 2.2-fold upon treatment with 5 ng/ml TGF-β1 for 24 hours, and 4-fold after the same treatment for 48 hours (Fig. 9B).
Figure 8. Time-dependent effects of IL-1β on ADAMTS-1 in decidual stromal cells. A, QC-PCR analysis of ADAMTS-1 mRNA levels in decidual cells cultured in the presence of IL-1β (100 IU) for 0, 6, 12, 24, or 48 hours (lanes 1-5, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrograph. The absorbance values obtained from three independent studies are represented (mean ± SEM, n=3) in the bar graphs below (*, P<0.001 vs. untreated control). B, Western blot analysis of ADAMTS-1 expression in protein extracts (20μg) prepared from decidual stromal cells cultured in the presence of IL-1β (100 IU) for 0, 6, 12, 24, or 48 hours (lanes 1-5, respectively). The data derived from this analysis as well as those from two other studies were standardized to the mean obtained from the untreated control and are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. 0 hours control).
Figure 9. Time-dependent effects of TGF-β1 on ADAMTS-1 expression in decidual stromal cells. A, QC-PCR analysis of ADAMTS-1 mRNA levels in decidual cells cultured in the presence of TGF-β1 (5 ng) for 0, 6, 12, 24, or 48 hours (lanes 1-5, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrograph. The absorbance values obtained from three independent studies are represented (mean ± SEM, n=3) in the bar graphs below (*, P<0.001 vs. untreated control). B, Western blot analysis of ADAMTS-1 expression in protein extracts (20μg) prepared from decidual stromal cells cultured in the presence of TGF-β1 (5ng) for 0, 6, 12, 24, or 48 hours (lanes 1-5, respectively). The data derived from this analysis as well as from two other studies were standardized to the mean obtained from the untreated control and are represented (mean ± SEM, n=3) in the bar graphs (*, P<0.001 vs. 0 hours control).
3.4: Attenuation of cytokine-modulated ADAMTS-1 mRNA and protein levels in human decidual stromal cells using monoclonal antibodies directed against IL-β1 or TGF-β1

The IL-1β-mediated increase in ADAMTS-1 mRNA levels in human decidual stromal cells was abolished by the addition to the culture medium of a monoclonal antibody directed against this cytokine. IL-1β at 100 IU/ml together with anti-IL-1β antibody at 1 μg/ml induced approximately a 1.8-fold decrease in ADAMTS-1 mRNA compared to the control treatment (IL-1β at 100 IU/ml), while IL-1β at 100 IU/ml together with anti-IL-1β antibody at 2 μg/ml induced approximately a 2.1-fold decrease in ADAMTS-1 mRNA compared to the control treatment (IL-1β at 100 IU/ml) (Fig. 10A). In accord with these fold-decreases in mRNA, ADAMTS-1 protein expression was decreased 1.6-fold by 100 IU/ml of IL-1β together with anti-IL-1β antibody at 1 μg/ml, and 1.8-fold by 100 IU/ml of IL-1β together with anti-IL-1β antibody at 2 μg/ml (Fig. 10B).

The decrease in ADAMTS-1 mRNA transcription levels observed in decidual stromal cells cultured in the presence of TGF-β1 alone was inhibited by the simultaneous addition of antibody directed against this growth factor. TGF-β1 at 5 ng/ml together with anti-TGF-β1 antibody at 10 μg/ml induced approximately a 2.1-fold increase in ADAMTS-1 mRNA compared to the control (TGF-β1 at 5 ng/ml) (Fig. 11A). A similar fold-increase (1.8-fold) in ADAMTS-1 protein expression was observed under these conditions (Fig. 11B).
Figure 10. Effects of anti-IL-1β neutralizing monoclonal antibody on ADAMTS-1 mRNA and protein levels in decidual stromal cells cultured in the presence of IL-1β. 

A, QC-PCR analysis of ADAMTS-1 mRNA levels in untreated decidual cells (lane 1) or cells cultured in the presence of a fixed amount of IL-1β (100 IU) and increasing amounts (0, 1 μg or 2μg) of the anti-IL-1β antibody for 24 hours (lanes 2-4). The sizes of the resultant target and internal standard PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrograph. The absorbance values obtained from three independent studies are presented (mean ± SEM) in the bar graphs below. **, P<0.01; *, P<0.001 (vs. treatment with IL-1β alone). 

B, Western blot analysis of ADAMTS-1 expression levels in protein extracts (20μg) prepared from untreated decidual stromal cells (lane 1) or cells cultured in the presence of a fixed amount of IL-1β (100 IU) and increasing amounts (0, 1 μg or 2μg) of the anti-IL-1β antibody for 24 hours (lanes 2-4). Data are shown as the mean of three independent studies ± SEM in the bar graphs. **, P<0.01; *, P<0.001 (vs. treatment with IL-1β alone).
Figure 11. Effects of anti-TGF-β1 neutralizing monoclonal antibody on ADAMTS-1 mRNA and protein levels in decidual stromal cells cultured in the presence of TGF-β1. A, QC-PCR analysis of ADAMTS-1 mRNA levels in untreated decidual cells (lane 1) or cells cultured in the presence of a fixed amount of TGF-β1 (5 ng) and increasing amounts (0 or 10 μg) of the anti-TGF-β1 antibody for 24 hours (lanes 2-3). The sizes of the resultant target and internal standard PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrograph. The absorbance values obtained from three independent studies are presented (mean ± SEM) in the bar graphs below. ***, P<0.05 (vs. treatment with of TGF-β1 alone). B, Western blot analysis of ADAMTS-1 expression levels in protein extracts (20 μg) prepared from untreated decidual stromal cells (lane 1) or cells cultured in the presence of a fixed amount of TGF-β1 (5 ng) and increasing amounts (0 or 10 μg) of the anti-TGF-β1 antibody for 24 hours (lanes 2-3). Data are shown as the mean of three independent studies mean ± SEM in the bar graphs. ***, P<0.05 (vs. treatment with of TGF-β1 alone).
A

![Image of gel electrophoresis showing bands at 620 bp and 321 bp.]

**Target / standard cDNA**

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<th>5</th>
<th>5</th>
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<tbody>
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<td>Anti TGF-β1 (ug/ml)</td>
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<td>0</td>
<td>10</td>
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</tbody>
</table>

B

![Image of Western blot showing a band at 110 kDa.]

**Relative Protein Expression**

<table>
<thead>
<tr>
<th>Anti TGF-β1 (ug/ml)</th>
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<th>0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti TGF-β1 (ug/ml)</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>
PART IV. DISCUSSION AND CONCLUSION

4.1: Discussion:

Multiple ADAMTS subtypes were detected in first trimester human decidual tissues. To date, the biological significance of ADAMTS-1 to -12 expression in this dynamic tissue remains to be elucidated.

Here, I report that ADAMTS-1 is expressed in primary cultures of stromal cells isolated from first trimester human decidual tissues. I have also determined that IL-1β increased ADAMTS-1 mRNA and protein levels, whereas TGF-β1 decreased ADAMTS-1 mRNA and protein levels in these primary cell cultures in both a dose- and time-dependent manner.

ADAMTS-1 mRNA transcripts are present, albeit at low levels, in a wide variety of adult human tissues including term placenta and non-pregnant uterine tissues (Bornstein et al., 1994; Gantt et al., 1997; Magnetto et al., 1998; Zimmermann et al., 2001). ADAMTS-1 expression has been detected in uterine tissues of pregnant mice (Shindo et al., 2000). The present study is the first demonstration that ADAMTS-1 is expressed in the human endometrium during early pregnancy. However, the role of this novel metalloproteinase in the morphological and functional differentiation of the mouse uterus remains unclear. Endometrial tissues of ADAMTS-1-null mice have been shown to develop large cysts and are capable of undergoing decidualisation (Shindo et al., 2000; Mittaz et al., 2004).
However, both studies reported reduced pregnancy rates in these gene-knockout mice, suggesting that ADAMTS-1 plays a critical role during early pregnancy. Although it is still not known whether ADAMTS-1 expression is spatiotemporally regulated in the human endometrium during the menstrual cycle, its expression in both the human placenta and decidua strengthen my hypothesis that this novel metalloproteinase mediates, at least in part, the tissue remodeling events that occur at the maternal-fetal interface during pregnancy.

ADAMTS-1 is a secreted, multifunctional protein with a multidomain structure. It possesses an amino terminal prodomain, a catalytic domain, a disintegrin-like domain, and an ECM binding domain composed of a central thrombospondin (TSP) type 1 motif, a spacer region and 3 TSP-like motifs (Tang et al., 2000) (refer to overview, sections 1.6.1). The proteolytic (catalytic) domain of ADAMTS-1 has been associated with the selective degradation of the ECM under normal and pathological conditions. For example, in the ovary, ADAMTS-1 has been shown to have a non-redundant role in the degradation of the follicle wall during ovulation and it facilitates the transport of the oocyte through the oviduct by mediating, at least in part, the expansion of the cumulus-oocyte complex (Russell et al., 2003).

Similarly, the proteolytic activity of ADAMTS-1 has been associated with local tissue invasion in cancer (Masui et al., 2001), and with the degradation of cartilage in osteoarthritis (Kuno et al., 2000). The development of inflammation associated with these two diseases or in response to trauma has also been linked to the enzymatic capacity of this
protein (Kuno et al., 1997). ADAMTS-1 has also been shown to have potent angio-inhibitory properties \textit{in vitro} (Vazquez et al., 1999). However, increased ADAMTS-1 expression had no significant effect on the vascularisation of colon carcinomas \textit{in vivo} (Masui et al., 2001). Furthermore, ADAMTS-1 appears to be necessary for the formation of the adrenomedullary capillary network during embryonic development (Mittaz et al., 2004). Collectively, these observations suggest that the angio-inhibitory/angiogenic activities of ADAMTS-1 are dependent upon the tissue, developmental stage, or the disease state. To date, the biological significance of ADAMTS-1 in the human decidua remains unclear although remodeling of the ECM and maternal vasculature are both key developmental events underlying the establishment and maintenance of pregnancy in humans.

Consistent with our observations, IL-1 has been shown to increase ADAMTS-1 in mouse colon carcinoma cells (Kuno et al., 1997) and in rat motor neurons (Sasaki et al., 2001). In contrast, the presence of this cytokine has been shown to significantly reduce ADAMTS-1 mRNA levels in human articular chondrocytes (Wachsmuth et al., 2004). In the present study, I have demonstrated that IL-1β increases and TGF-β1 decreases ADAMTS-1 mRNA and protein expression in human decidual stromal cells. It is not clear whether the regulatory actions of IL-1β and TGF-β1 on ADAMTS-1 mRNA and protein levels in human decidual cells are mediated directly or indirectly, possibly via alterations in the expression of other cytokines. For example, IL-1 has been shown to increase the expression of IL-6 and IL-10 in human endometrium stromal cells \textit{in vitro} (Tabibzadeh et al., 1989), suggesting that IL-1 regulates a pro-inflammatory cascade in the human decidua.
that results in the activation of proteolytic mechanisms operating at the maternal interface.

Similarly, TGF-β1 has been shown to decrease mRNA and protein expression levels of IL-8 in human endometrial stromal cells in vitro (Arici et al., 1996). IL-8 is a potent angiogenic factor that has reduced immunoreactivity in the human decidua during early pregnancy (Lockwood et al., 2004).

Progesterone (P4) is a key regulator of decidualisation (Irwan et al., 1989). This gonadal steroid has also been shown to regulate ADAMTS-1 in the rodent ovary (Robker et al., 2000). Mice null-mutant for the P4 receptor or those treated with the P4-antagonist, epostane (Espey et al., 2000), fail to express this ADAMTS subtype in the preovulatory follicle. However, computer-based searches have failed to detect a P4 response element in the putative promoter region of the ADAMTS-1 gene (Mittaz et al., 2004) suggesting that the regulatory effects of this gonadal steroid on ADAMTS-1 expression are not mediated through direct interaction. IL-1 and TGF-β1 have been shown to both promote and inhibit the biological actions of P4 on the human endometrium. For example, TGF-β1 has been shown to act in concert with P4 to promote decidualisation but it opposes the stimulatory actions of this gonadal steroid on the expression of enkephalinase in endometrial stromal cells in vitro (Casey et al., 1996). Likewise, IL-1β has been shown to inhibit decidualisation (Kariya et al., 1991), but promotes secretion of the biochemical marker of decidualisation, insulin-like growth factor binding protein (Frost et al., 2000).

In this study, a single ADAMTS-1 protein species of approximately 110 kDa was observed corresponding to the proform of ADAMTS-1 which was consistently expressed in
all of the decidual stromal cell cultures examined and was differentially regulated by IL-1β and TGF-β1 in a dose- and time-dependent manner. Biochemical studies indicate that the ADAMTS-1 protein is subject to one distinct post-translational cleavage event which results in the generation of a distinct bioactive fragment of approximately 87 kDa (Longpre et al., 2004).

In support of my findings, only the proform of ADAMTS-1 was detected in cultures of human chondrocytes (Wachsmuth et al., 2004). Differences in the detection of the distinct forms of ADAMTS-1 may be attributed to variations in the epitope recognized by the distinct ADAMTS-1 antibodies available or the protein extraction methods used in these studies. Alternatively, the generation of these distinct fragments, which appear to have distinct biological functions, may be cell/tissue specific and/or be dependent upon the presence of other proteolytic factors, particularly MMP-2, MMP-8 or MMP-15, which have shown to be capable of cleaving ADAMTS-1 in vitro (Rodriguez et al., 2000).
4.2: Conclusions

In this study, I have shown that ADAMTS-1 to -12 subtypes are expressed in human decidua. Furthermore, I have demonstrated that ADAMTS-1 mRNA expression is differentially regulated by the cytokines IL-1β which induces ADAMTS-1 expression, and TGF-β1 which inhibits ADAMTS-1 expression.

In view of the expression of ADAMTS subtypes in human decidua and the regulatory effect of IL-1β and TGF-β1 on ADAMTS-1, it is possible that other ADAMTS subtypes may also be regulated by IL-1β and TGF-β1 in primary cultures of human decidual stromal cells, and play key roles in the endometrial ECM remodeling events that occur at the maternal-fetal interface during early pregnancy.

The studies described in this thesis contribute to our understanding of the basic cell biology of the ADAMTS gene family. In addition, my findings provide some initial insight into the cellular mechanisms underlying the ECM remodeling events that occur in the human endometrium in preparation for pregnancy.
4.3: Future studies

*Identification of the regulatory effects of IL-1β and TGF-β1 on ADAMTS-2 to -12 mRNA and protein levels in human decidual stromal cells.*

To determine whether other members of ADAMTS family (ADAMTS-2 to -12) are regulated by IL-1β and TGF-β1, QC-PCR and Western blotting analyses could be used to determine their mRNA and protein levels in decidual stromal cells from first trimester human decidual tissues.

*Cellular localization of the multiple ADAMTS subtypes present in human first trimester decidua*

In the present study, I examined the repertoire of ADAMTS present in the decidual tissues using a semiquantitative RT-PCR strategy. The RT-PCR approach was chosen because there are currently a limited number of antibodies available for the direct detection of members of the ADAMTS gene family. As antibodies specific for the ADAMTS subtypes identified in human decidual tissues and cells become available, the present study can be extended to determine ADAMTS protein expression and cellular localisation using Western blot and immunohistochemistry analysis.
Identification of the ADAMTS substrates present in human decidual tissues.

To better understand the functional and molecular actions of the different ADAMTS subtypes, it is necessary to identify the various ADAMTS substrates present in the extracellular matrix of human decidua during early pregnancy.
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