GONADAL STEROIDS REGULATE ADAMTS-1 EXPRESSION IN HUMAN ENDOMETRIAL STROMAL CELLS IN VITRO

by

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M.D., Capital University of Medical Sciences, China, 1993

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

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Reproductive and Developmental Science)

UNIVERSITY OF BRITISH COLUMBIA

April 2006

ABSTRACT

Gonadal steroids are regulators of the ECM remodeling events that occur in the human endometrium during each menstrual cycle. The ADAMTS represent a novel family of MMPs, the best characterized of which is the initially identified member, ADAMTS-1. ADAMTS-1 has recently been found to be spatiotemporally expressed in the human endometrium during the menstrual cycle with mice null-mutant for this ADAMTS subtype also exhibiting endometrial dysfunction. To date, the factors capable regulating ADAMTS-1 in the human endometrium have not been identified. In view of these observations, I hypothesized that ADAMTS-1 plays a central role in the steroid-mediated remodeling events that occur in the human endometrium during each reproductive cycle. In the studies presented in this thesis, I have examined the ability of the gonadal steroids, progesterone (P4), 17β-estradiol (E2) or the non-aromatisable androgen, dihydrotestosterone (DHT), alone or in combination to regulate ADAMTS-1 mRNA and protein levels in primary cultures of human endometrial stromal cells in a time- and concentration-dependent manner. In addition, I determined whether the anti-steroidal compounds, RU486 (an antiprogestin), ICI 182, 780 (an anti-estrogen) or hydroxflutamide (an anti-androgen) were capable of inhibiting the regulatory effects of these gonadal steroids on stromal ADAMTS-1 levels. Real-time PCR and Western blotting revealed that P4 and DHT increased ADAMTS-1 expression levels whereas E2 alone had no regulatory effect on the expression levels of this ADAMTS subtype in these primary cell cultures. A combination of DHT and P4 potentiated the increase in the levels of the ADAMTS-1 protein species present in these cell cultures whereas E2 was capable of attenuating the stimulatory effects of both P4 and DHT on stromal ADAMTS-1

mRNA and protein expression levels. In contrast, RU486 and hydroxyflutamide specifically inhibited the increase in ADAMTS-1 expression levels mediated by P4 and DHT, respectively. In summary my studies, demonstrate that the regulation of ADAMTS-1 mRNA and protein expression levels in human endometrial stromal cells by gonadal steroids involves a complex interplay between progestins, estrogens and androgens.

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LIST OF ABBREVIATION

ADAM	A Disintegrin and Metalloproteinase
ADAMTS	A Disintegrin and Metalloproteinase with TromboSponding repeats
E2	17β-estradiol
ER	Estrogen Receptor
P4	Progesterone
PR	Progesterone Receptor
T	Testosterone
DHT	5α-dihydrotestosterone
AR	Androgen Receptor
ECM	Extracellular Matrix
MMP	Matrix Metalloproteinase
MT-MMP	Membrane-type Matrix Metalloproteinase
TIMPs	Tissue inhibitors of MMPs
uPA	urokinase plasminogen activator
tPA	tissue-type plasminogen activator
PAI	Plasminogen activator inhibitor
SEM	Standard error of mean
PRL	Prolactin
IGFBP-1	Insulin-like growth factor binding protein-1
PRM	Progesterone receptor modulator
HSPG	Heparan sulfate proteoglycan
TSP	Thrombospondin
SVMP	Snake venom metalloprotease
PCR	Polymerase Chain Reaction
cDNA	Complementary Deoxyribonucleic Acid
DMEM	Dulbecco's Modifided Eagle Medium
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
FBS	fetal bovine serum
ANOVA	Analysis of variance

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to Dr. Colin D. MacCalman, my research supervisor, for providing excellent mentorship to me these past years; for guiding my research with understandable and clear direction through the various stages of this work, being so thorough and concern in supervising the preparation of this thesis. Truly speaking, without his brilliant ideas, personal guidance and patient instruction, it never would have been possible to complete these projects in first place. Secondly, I want thank Dr. Peter C.K. Leung, the Director of Reproductive and Developmental Sciences, who has always offered the most valuable and professional advice to me. I also truly appreciated his support throughout these studies.

I want to take this opportunity to thank my supervisory committee: Dr. Peter McComb (chairman), Dr. Peter C.K. Leung, Dr. Timothy Rowe and Dr. Beth Taylor for overseeing my research progress and give me valuable and constructive suggestion. I would like to express my most sincere appreciation to Dr. Catherine J. Pallen for being my external examiner for her most valuable time and comments on this thesis.

I would also like to thank Dr. Hua Zhu in Dr. MacCalman's lab for her most valuable technical support, advice, comments and friendship. As well I would like to take this opportunity to express my appreciation to my collegues: Dr. Shuko Murakami, Alexander Beristain, York H. Ng and other lab members for their constant support and friendship during the time we have spent together.

I would also like to express my thanks to the members in Oncology division for their ongoing support on the tissue collection.

Finally, I am forever indebted to my parents for their nurturance, for giving me encourage and confidence during my growing up. I would also like to thank my husband and my son for their sharing my burden, for their understanding and the love and happiness they gave to me. Here I dedicate this thesis to my family by expressing my sincere appreciation to the people I deeply loved.

PART 1 OVERVIEW

1.1 Background

Pregnancy loss is major health concern facing reproductive medicine in the 21st century. Approximately 35% of pregnancies never come to term and approximately 10% of couples trying to establish a family suffer from severe fertility problems (Stephenson 1996; Paria et al., 2002). It is projected that these clinical problems will increase sharply in the next decade as women in developed countries continue to delay child-bearing. (Ventura et al., 2003).

Uncertainty about the underlying causes of infertility associated with implantation failure often leads to open-ended empirical treatments that can create significant emotional and financial burdens for women, families and the health care system (El-Toukhy, 2002). Furthermore, notwithstanding the recent advances made in assisted reproductive technologies, the success rate of in vitro fertilization and embryo transfer (IVF-ET) rarely exceeds 25% (Nygren and Andersen, 2002). Our inability to significantly improve the pregnancy rate in these women provides further evidence that current clinical practices may have outpaced our basic understanding of the biology of embryonic implantation and placentation.

A better understanding of the molecular determinants that create a uterine environment capable of supporting pregnancy will allow us to pinpoint critical aspects of this developmental process that are particularly vulnerable to failure.

1.2 Structural Development of the Endometrium

The human endometrium undergoes cyclic remodeling in preparation for pregnancy (Noyes et al., 1950). After menstruation, the endometrium regenerates to produce a dense cellular stroma containing narrow tubular glands and small blood vessels. Immediately after ovulation, a change in epithelial cell morphology can be observed with larger gland profiles and the appearance of basal glycogen masses in these endometrial cells. In contrast, there is little change 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; with an embryo present, P4 levels will continue to increase, leading to decidualisation of the stroma. Alternatively, in the absence of pregnancy, P4 levels will fall to produce a late secretory endometrial phenotype, followed by menstrual shedding.

The endometrium is only receptive to the implanting embryo at a certain stage of the menstrual cycle, named the "window of implantation" (ADAM et al., 1956). At other time period of the menstrual cycle, the endometrium is "non-receptive". The putative "window of implantation" in humans is believed to span cycle days 20-24 and involves the luminal epithelium and subsequently the endometrial stroma (Adam et al. 1956; Nikas,

1999; Wilcox et al., 1999). This receptive period is associated with distinct morphological and molecular changes in the luminal epithelium of the endomtrium. In particular, epithelial dome-like structures (pinopodes), that are believed to mediate the attachment of the embryo to the luminal epithelium, appear at the implantation site (Lindenberg, 1991). The expression of several molecules in this endometrial cell layer including carbohydrate epitopes, H-type 1 antigen, heparan sulfate proteoglycan, mucins, integrin subunits (particulary $\alpha v\beta 3$ and $\alpha 4\beta 1$) and the trophin-bystin/tastin complex has also been found to be temporally regulated in this endometrial cell layer, thereby 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 exhibits histological changes that represent the earliest cascade of differentiative events leading to decidualisation (Noyes et al., 1950), a key cellular event in implantation. Focal areas of edema appear in which the density of stromal cells is reduced. As a result, blood vessels in these areas are more obvious, although no overt vascular differentiation is yet evident. Other areas of the stroma are still densely populated with elongated mesenchymal cells. As in other phases of the menstrual cycle, but now becoming more apparent, 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 extensive, though more densely cellular areas also still persist. At this time, vascular differentiation occurs to produce prominent spiral arterioles surrounded by a cuff of pseudo-decidual cells, enlarged stromal cells that resemble the decidual cells of pregnancy.

Decidualisation of the endometrium involves the differentiation of the stromal cells that acquire distinct morphological and functional features (Noyes et al., 1950; Wynn, 1974). Morphological decidualisation is expressed histologically by a change from a spindle to a polyhedral cell shape with an increase in cell size, in conjunction with, an extensive development of the organelles involved in protein synthesis and secretion, and by the appearance of desmosomes and gap junctions (Lawn et al., 1971; Wynn, 1974; Jahn et al., 1995). Functionally, decidualisation is associated with the onset of prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) secretion (Maslar et al., 1979; Lala et al., 1984). A significant population of bone-marrow derived cells, amounting to which include large granular lymphocytes (LGLs), macrophages and to a lesser extent, T cells are also now present and account for over 40% of cells of deciduas (Starkey et al., 1988; Bulmer et al., 1990). The LGLs are believed to arise 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 observed between these bone marrow-derived cells and resident decidual cells (Aplin et al., 1988). Most cells have also been detected in the human decidua (Marx et al., 1999).

The diverse populations of cells that constitute the decida allows this dynamic tissue to fulfill paracrine, nutritional, and immunoregulatory functions throughout pregnancy (Lala and Kearns, 1984) In addition, the decidua plays a key embryoregulatory role by virtue of its intrinsic ability to regulate the invasion of trophoblastic cells into the underlying maternal tissues and vasculature during early pregnancy (Bischof et al., 2000). The depth

of trophoblast invasion is precisely controlled by the decidua and errors have extreme consequences on the health of the mother and fetus (Cross et al., 1994; Paria et al., 2002)

1.3 Endometrial Cell Model Systems

Progress in our understanding of the development of uterine environment that will support pregnancy in humans has been hampered by the fact that *in vivo* human experimentation is not ethically feasible and the morphological differences between the human placenta and the process of decidualisation and that of experimental and domestic animals (Leiser and Kaufmann, 1994). Consequently, most of our information regarding these two inter-related developmental processes relied on histological studies of hysterectomy or term placental tissue specimens (Hertig, 1967; Hamilton and Grimes, 1970; Pijnenborg et al., 1980). More recently, several in vitro model systems have been developed and used to examine the biochemical and cellular mechanisms underlying the development, maintenance and regression of the human endometrium.

The ability of gonadal steroids to regulate the cyclic remodeling processes that occur in the endometrium was first demonstrated using ovariectomized rodent model systems (Psychoyos, 1976). However, in contrast to the rat and mouse, embryonic implantation in the human occurs at a time when the stroma is not yet decidualised (Noyes et al., 1950; Hertig, 1967). Consequently, the molecular and biochemical mechanisms underlying the differentiation of the human endometrium have been determined using cultures of endometrial explants (Bentin-Ley et al., 1994), endometrial carcinoma cell lines

(Somkuti et al., 1997) and primary cultures of cells isolated from endometrial tissue specimens obtained from women with a variety of medical conditions and at all stages of the menstrual cycle and early pregnancy (Irwin et al., 1989; Fernandez-Shaw et al., 1992; Shiokawa et al., 1996).

In vitro models include primary cultures of the stromal and glandular epithelial cells which can be enzymatically isolated and maintained in culture (Irwin et al., 1989; Fernandez-Shaw et al., 1992; Shiokawa et al., 1996). Bone marrow-derived cells and vascular cells have also been recovered using similar enrichment procedures (Starkey et al., 1988). In view of the likelihood of intercellular communication via soluble mediators (Wegmann et al., 1993), it is important to define and characterize the cells present in the culture models. The differentiation state of the cell cultures is also an important variable. The addition of gonadal steroids to the culture medium of endometrial stromal cells stimulates decidualisation as determined by morphological differentiation and the production of biochemical markers including prolactin, laminin and IGFBP-1 (Irwin et al., 1989). In contrast, the removal of gonadal steroids from this model culture system mimics many of the molecular and biochemical events associated with the late luteal phase and menstruation (Salamonsen et al., 1997).

1.4 Cellular Mechanisms Underlying Endometrial Remodeling

1.4.1 Gonadal Steroids

The human endometrium is a dynamic tissue that undergoes well-defined cycles of proliferation, differentiation, and shedding in response to the prevailing endocrine and paracrine environment. It has been well established that 17β-estradiol (E2) promotes cellular proliferation in the stroma and glandular epithelium of the endometrium, particularly during the proliferative phase of the menstrual cycle. Progesterone (P4) in turn, is believed to act upon the E2-primed endometrium, thereby initiating glandular secretion and the differentiation of stromal cells into decidual cells during the secretory phase of the menstrual cycle (Noyes et al., 1950; Clark et al., 1980).

Steroids interact with their target organs via their corresponding and specific nuclear receptors, E2 binds to estrogen receptors (ER), P4 binds to progesterone receptors (PR) and androgen binds to androgen receptors (AR). The receptors vary temporally and spatially across the menstrual cycle (Critchley et al., 2001; Snijder et al., 1992; Garcia et al., 1988; Lessey et al., 1988).

The PR is composed of two hormone binding proteins, designated PRA and PRB (Truss and Beato 1993). These two proteins are encoded by a single gene under the control of distinct promoters, each of which generates distinct PR mRNA transcripts (Kastner at al., 1990). PRA and PRB are both capable of binding progestins and interacting with steroid

response elements (HREs). However, there is increasing evidence to suggest that they are functionally distinct. For example, in transfection studies these two proteins have different abilities to activate progestins responsive promoters (Tora et al. 1989l; Vegeto et al.1993). These differences were promoter- and cell-specific suggesting that cellular responsiveness to progestins may be modulated via alterations of the ratio of PRA and PRB expression. Although PRB tends to be a stronger activator of target genes, PRA can act as a dominant repressor of PRB (Tung et al. 1993; Vegeto et al. 1993). These observations suggest that high PRA expression may result in reduced progestin responsiveness and that PRA and PRB may thus be a repressor and activator, respectively.

In general, PR expression levels are believed be regulated by E2 and P4 which increase and decrease the levels of this receptor in target tissues, respectively (Levy et al. 1980). In agreement with these observations, E2 has been shown to up-regulate PR whereas P4 has been shown to decrease the levels of both protein isoforms in isolated human glandular epithelial cells (Eckert and Katzenellenbogen 1981; Evans and Leavitt 1980; Katzenellenbogen 1980; Kreitmann et al. 1979). In contrast, P4 was shown to be capable of coordinately up-regulate the expression levels of PRA and PRB in human endometrial stromal cells *in vitro* (Tseng and Zhu 1997).

Two structurally related subtypes of ER, commonly known as ERα and ERβ, have been identified in human, as well as in other mammals (Green et al., 1986; Kuiper et al., 1996). A second isoform of the ER has been reported in certain E2-responsive tissues in rat (Kuiper et al. 1996) and human (Mosselman et al. 1996). This isoform, termed ER-β, is

highly homologous to the α-isoform of the receptor, particularly in the DNA-binding and ligand-binding domains (Kuiper et al. 1997; Kuiper et al. 1996). In ligand binding assays ER-β has been shown to bind E2 with an affinity and specificity that is similar to ER-α (Kuiper et al. 1996). ER-β is able to activate transcription of E2-responsed element-containing reporter gene constructs (Kuiper et al. 1996). Furthermore, homodimers and heterodimers of these two ER isoforms are capable of activating transcription, in an E2-dependent manner, from reporter gene constructs containing estrogen response elements. The activation of these gene constructs was more efficient with homodimers of ER-α (Pettersson et al. 1997). However, recent studies have demonstrated that these two ER subtypes have opposite regulatory modes to the natural hormone from the same DNA response element in transfection studies (Paech et al. 1997). In particular, with ER-α, E2 activates transcription, whereas with ER- β, E2 inhibits transcription at AP-1 site (Paech et al. 1997).

ERα and PR are highly expressed in the glandular epithilial and endometrial stroma cells of the human endometrium during proliferative phase of the menstrual cycle. There is a marked decrease in ERα levels as the menstrual cycle enters the secretory phase (Snijders et al., 1992; Mertens et al., 2001; Taylor et al., 2005). In contrast, ERβ levels are low in proliferative endometrial glands with maximum levels being detected in this cellular compartment in secretroy endometrium. ERβ was also been detected in endometrial endothelial cells, suggesting E2 may also act directly upon the vasculature of this dynamic tissue (Critchley et al., 2000; Lecce et al., 2001).

There is also increasing evidence to suggest that androgens play an integral role in the cyclic remodeling events that occur in the endometrium under normal and pathological conditions. For example, the concentration of androgens in the human endometrium exceeds those in plasma (Guerrero et al., 1975; Vermeulen-Meiners et al., 1988). In a clinical setting, chronic hyperandrogenism associated with poor reproductive outcome, polycystic ovarian syndrome (PCOS), there is an increase in the level of circulating androgen and an elevation of AR expression in endometrium (Apparao et al., 2002). It has also been reported that, in women with recurrent miscarriage, high androgen concentrations may specifically act in the human endometrium (Okon et al., 1998). The direct biological actions of androgens of are mediated by the spatiotemporal expression of AR in the endometrium. In particular, AR levels are low in the glandular epithelium and stromal cells of the proliferative endometrium and increase in both cellular compartments as the menstrual cycle progresses to the secretory phase (Horie et al., 1992; Slayden et al., 2001; Burton et al., 2003; Kato and Seto 1985).

There is increasing evidence to suggest that the biological actions of androgens on endometrial tissues vary between species and can mimic both E2 and P4. In the rodent uterus, androgens primarily have an estrogenic effect. For example, testosterone (T) and the non-aromatisable androgen, 5α-dihydrotestosterone (DHT), which cannot be aromatized into estrogen, markedly increased uterus weight and the height of the uterine luminal epithelialum, and modulate biological events simply mimicking estrogen. These effects were not prevented by the co-adminsistration of antiestrogens. However, DHT cannot induce uterine growth in ERα knockout mice. Global gene profiling of E2- and

DHT-regulated gene programs in the rat endoemtrium demonstrates suggest that these two gonadal have both overlapping and distinct regulatroy effects on genes underlying the morphological and functionnal maturation of this dynamic tissue including those associated with (1) protein synthesis, maturation degradation and secretion (2) intracellular signaling and signal transduction (3) tissue growth and remodeling and (4) metabolism and metabolite transport (Gonzalez-Diddi et al., 1972; Nantermet et al., 2005). However, DHT was also capable of maintaining decidualisation in the mouse although it could not substitute for P4 in the priming of the mouse uterus suggesting a dual role of androgens in rodent endometrim, dependent upon the stage of the reproductive cycle (Zhang et al., 1996).

In contrast to the rodent, androgens have a "purely" progestogenic effect on the human endometrium For example, T and DHT both induce of prolactin (PRL) production, a biochemical marker of decidualisation, in a similar manner to that observed in cells cultured with P4. Furthermore, a combination of P4 and T enhanced prolactin (PRL) production in these cell cultures compared to those cultured in the presence of either steroid alone. Flutamide, a specific androgen receptor blocker was capable inhibit T and DHT but not P4 induced PRL secretion (Narukawa et al., 1994). This further suggested that androgen play important roles in human endometrial differentiation. Collectively, these observations suggest that progestins and androgens have independent but cooperative biological actions on endometrial stromal cells differentiation in vivo and in vitro.

1.4.2 Antisteroidal Compounds

Over the last decade, antisteroidal compounds have replaced surgery as the first choice for the management/tratement of steroid-based medical conditions, including cancer and endometriosis as well as facilitating better control of menstruation and fertility. In addition to their use in the clinical setting, antisteroidal compounds have become useful tools for the study of the molecular and cellular mechanisms underlying the biological action of gonadal steroid hormones under normal and pathological conditions.

Anti-progestins

The first effective and best characterized antiprogestin is RU486 (mifepristone) (Chwalisz et al., 2000). Several other antiprogestins have subsequently been developed, such as onapristone, which have greater potency and higher specificity for the progesterone receptor (PR) (Chwalisz et al., 2000; Chabbert-Buffet et al., 2005). In addition, mixed antagonists commonly known as mesoprogestins or progesterone receptor modulators (PRMs) have been developed. PRMs can block the actions of P4 but in the absence of this gonadal steroid can act as agonists. Both antiprogestins and PRMs have many potentially important clinical uses and chronic low dose treatment with antiprogestins has been proposed for the management of aberrant endometrial bleeding, endometriosis, breast cancer, and contraception.

RU486 has been shown to act as an effective antigestional agent when administered during the luteal phase of the menstrual cycle and as an abortifacient when administered during pregnancy (Gobello, 2006). In particularly, the administration of RU486 during the early luteal phase of menstrual cycle delays the development of a secretory endometrium without affecting the function of corpus luteum or the length of the menstrual cycle (Gemzell-Danielsson, et al., 1994). RU486 binds to the progesterone receptor (PR) with a higher affinity than its naturally occurring ligands (Baulieu, 1997). RU486 prevents P4 binding to the PR, which in turn, results in P4 being eliminated from the cell or metabolished in situ. In addition, it is believed that RU486 also decreases PR levels in the human endometrium (Zaytseva et al., 1993).

Anti-estrogens

Pharmacologic groups of compounds that inhibit or modify estrogens are classified as anti-estrogens. Some of these are gonadotrophin releasing hormones (GnRH) analogs and aromatase inhibitors that inhibit estrogen synthesis. Another anti-estrogenic drug group is estrogen receptor blockers (Williams et al., 1996). Clomiphene and tamoxifen citrate are synthetic nonsteroidal type-I anti-estrogenic compound which competitively block estrogen receptors with a combined antagonistic-agonistic effect. They partially inhibit the action of estrogen agonists, but due to their own agonistic properties, they also induce estrogenic responses (Hoffmann and Schuler, 2000). Thus in women, tamoxifen has anti-estrogenic activities on the mammary gland and agonistic effects on the uterus (Jordan, 1995).

ICI 182, 780, is a novel estrogen receptor "blockor", which acts as a 'pure' estrogen antagonist without estrogen-like, agonist activity (Kauffman et al., 1995; Wakeling et al., 1991; Dukes et al., 1992). It has the ER binding affinity approximately 100 times greater than that of tamoxifen and has no agonistic activity on target tissues including the endometrium and uterus. It has been studied by used in clinical trails as replacement of tamoxifen for the treatment of advanced, and tamoxifen-resistant breast cancer, as it has a longer duration of response and fewer side effects (Robertson, 2001; Hu et al., 1993). Additionally, ICI 182, 780 has been widely used experimentally to investigate the role of estrogen and other hormones on mammalian tissues and cells *in vivo* and *in vitro*.

Anti-androgens

Several groups of compounds have been described to have anti-androgenic properties, These include progestins, receptor binding anti-androgens, and aromatase inhibitors.

Flutamide/hydroxyflutamide is a pure androgen receptor blocker, which inhibits androgen uptake and nuclear binding by binding to the androgen receptor (Hoffmann et al., 2000). Clinically, flutamide has yielded good results in treating prostatic hyperplasia with few side-effects (Neri and Monahan, 1972). In addition, hydroxyflutamide has been shown to have anti-progestagenic activities in uterus, cervix, and hypothalamus in rats (Chandrasekhar 1991).

Aromatase inhibitors, such as formestane, exert their anti-androgenic effect by inhibiting the conversion of androgens to estrogens in peripheral tissues (Ito et al., 2000).

Although it is well established that gonadal steroids play pivotal roles in the cyclic remodeling events that occur in the endometrium in preparation for pregnancy, and that their biological actions can be counterbalanced by synthetic antisteroidal compounds, the molecular mechanisms underlying their biological action in this dynamic tissue at distinct stages of the menstrual cycle remain poorly characterized.

1.5 Remodeling of the Extracellular Matrix (ECM) of the Endometrium

Recent microarray array studies have demonstrated that the biological actions of gonadal steroids on the endometrium are mediated by the sequential and categorical activation of multiple gene programs with a wide variety of functions and which exhibit either distinct or overlapping expression patterns (Popovici et al., 2000). To date, the overall biological significance of these gene expression patterns in the developmental of a uterine environmet capable of supporting pregnancy remains to be elucidated. Consequently, remodelling of the endometrial ECM is still considered to be a key underlying event of the morphological and functional maturation of this dynamic tissue during the menstrual cycle and in pregnancy (Aplin et al., 1988; Iwahashi et al., 1996; Schatz et al., 1999)

Regeneration of the endometrium during the proliferative phase of the menstrual cycle involves the deposition of an ECM scaffold (Aplin et al., 1988; Mylona et al., 1995; Church et al., 1996). The undifferentiated stroma produces an ECM that has a classical mesenchymal composition; in particular collagens I, III, V, and VI and fibronectin (Fn) have all been shown to be present and there are periglandular deposits of tenascin that appear 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 anastomosing network in the intercellular spaces.

Changes in the ECM accompany the transition from undifferentiated stroma to decidua (Wynn, 1974; Wewer et al., 1985; Kisalus et al., 1987; Ruck et al., 1994). These include a decrease in type I collagen, fibronectin and laminin of endometrium significant decreased after implantation. In addition, the type IV collagen and laminin of epithelial basement membrane also remarkably declined during early pregnancy (Yamada et al., 2002). The decidual cell basement membrane is composed of laminins 2 and 4, type IV collagen, HSPG and BM-40 (Faber et al., 1986; Wewer et al., 1988 Church et al., 1996). 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 probably involved in the secretion of basement membrane components (Kislaus et al., 1988).

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 stabilization 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 and 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 a uterine environment into which trophoblast invasion may occur more readily (Aplin, 1991). The second is the appearance of laminins (Ln) 2 and 4 in association with the differentiating stromal cells (Church et al., 1996). As previous studies have demonstrated that laminin 2 is capable of mediating 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 apply to the migratory bone marrow-derived cells that 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 support 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).

Alterations in the composition in the endometrial ECM are coordinated with regulated changes in the expression levels of cell-matrix receptors on the cell surface. In particular, there is a marked increase in the endometrial levels of αv and β3 integrin subunits, members of the gene superfamily of calcium-dependent cell-matrix adhesion molecules, during the window of implantation in humans (Lessey, 2002) Aberrant expression of avb3 integrin subunits in the endometrium are associated with infertility and recurrent pregnancy loss (Lessey 1998).

1.5.1 Proteolytic Mechanisms Responsible for Endometrial ECM Remodeling

The majority of the tissue remodeling events that occur in the endometrium during 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 and Osteen, 2001). The decidua is also subject to further degradation, particularly the first trimester of pregnancy, by the invading trophoblast that have been shown to adopt similar cellular mechanisms for ECM degradation to those observed during tumour cell invasion and metastasis (Yagel et al., 1988; Strickland and Richards, 1992; Lala and Hamilton, 1996; Bischof et al., 2000).

Consequently, proteolytic enzymes have been assigned key roles in these develop process of tissue remodeling. In particular, urokinase plasminogen activator (uPA) and the matrix metalloproteinases (MMPs) have been shown work in concert or in cascades to degrade or process specific components of the endometrial ECM (Schatz et al., 1999; Fata et al., 2000; Curry et al., 2001). However, as aberrant expression or distribution of ECM components in the endometrial stroma has been associated with infertility and recurrent pregnancy loss (Bilalis et al., 1996; Jokimaa et al., 2002), it is important to define the full repertoire of proteinases expressed by these cells, their regulation, and ultimately, their individual contribution(s) to the development of a uterine environment that is capable of supporting pregnancy.

1.5.1.1 Plasminogen Activators and Their Inhibitors

The plasminogen activators are substrate-specific serine proteinases that mediate cleavage of plaminogen to plasmin, which exhibits a broad range of serine protease activity (Vasselli et al., 1991; Andreasen et al., 2000). The proteinase activator system includes the urokinase-type plasminogen activator (uPA), the tissue-type PA (tPA), and their endogenous inhibitors, PA inhibitor-1 and –2 (PAI-1 and PAI-2, respectively) and their common receptor, uPA receptor in the human endometrium.

uPA and tPA expression in the human endometrium are temporally expressed during the menstrual cycle with highest levels being detected during the secretory phase and early pregnancy (Casslen and Astedt, 1983; Koh et al., 1992). Similarly, PAI-1 expression

levels have also been shown to be high during the secretory phase and decline with the onset of menstruation (Koh et al., 1992). The withdrawal of P4 from the culture medium of endometrial stromal cells was found to increase uPA activity and concomitantly decrease PAI-1 expression levels in these cultures (Schatz et al., 1999). However, mice null mutant for uPA, urokinase receptor (uPAR), tPA, did not exhibit reduced fertility (Bugge et al., 1996), indicated that other proteinases also involved in this complex process.

1.5.1.2 Matrix Metalloproteinases and Their Endogenous Inhibitors

The matrix metalloproteinases (MMPs) are a large gene family of zinc-dependent proteinases that mediate a variety of tissue remodeling processes (Woessner, 1991; Fata et al., 2000). To date, 24 distinct members of the MMP gene family 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 though MT-MMP6) and a miscellaneous group that contains MMP-12, 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 their 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 and Werb, 2002). In general, 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, 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 proteloytic domain of the MMP subtypes with a stoichiometry of 1:1 (Woessner, 1991; Egebald and Werb, 2002). The unique structural properties of TIMP-3 however 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, TIMPs-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 (Ikenka 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 (Curry and Osteen, 2001, Fata et al, 2002; 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 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, MMP-9 but not MMP-1, MMP-7 have been detected in the decidua of early pregnancy whereas 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 the MMPs 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 in glandular epithelium and MMP-9 also 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 have been reported to be present in the stroma, MMP-7 in the glandular epithelium, and MMP-9 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 in the stroma, MMP-7 in the glandular epithelium and MMP-9 in monocytes, neutrophils and macrophages (Rodgers et al., 1993, 1994; Hampton et al., 1994, 1995; Marbaix et al., 1995; Kokorine 1996).

The human endometrium has also been shown to constitutively express TIMP-1, TIMP-2, and TIMP-3 whereas the expression of TIMP-4 in this dynamic tissue has not been examined (Fata et al., 2000, Osteen and Curry, 2001; Goffin et al., 2003). In contrast to

the MMPs, there appear to be only small fluctuations in the overall expression levels of TIMP-1, TIMP-2 and TIMP-3 expression in the endometrium during the menstrual cycle. However a localized increase in TIMP-1 mRNA and protein expression areas has been detected near small arteriolar and capillary tissue in the secretory endometrium and menstrual tissue suggesting that it may be focally regulated in the endometrial vasculature (Rodgers et al., 1993; Salamonsen and Woolley, 1996; Zhang and Salamonsen, 1997). Similarly, TIMP-2 mRNA and protein expression levels were found to be highest in the vasculature than glandular epithelium, stroma or decidua of early pregnancy (Hampton and Salamonsen 1994; Zhang and Salmonsen, 1999). TIMP-3 expression levels have also been shown to 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 (Zhang and Salmonsen, 1999; Goffin et al., 2003). Therefore, it appears that the regulation MMP expression levels in the human endometrium are two-fold involving large cyclic fluctuations in the epithelial and/or stromal cells of the endometrium and at small localized foci within these cellular compartments which occur through 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 (Marbaix et al., 1995; Osteen et al., 1994; 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 (Salamaonsen et al., 1997).

1.5.2 ADAMs (A Disintegrin And Metalloproteinase)

The ADAMs (A Disintegrin And Metalloproteinase) is a gene family of transmembrane proteins that contain a disintegrin and a metalloprotease domain. The metalloprotease domains can induce ectodomain shedding and cleave ECM protein. The disintegrin and cysteine-rich domain have adhensive activities. 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 signalling 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 and white, 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 Heparin-binding epidermal growth factor (HB-EGF) from cultured cells. ADAM-10 acts as a sheddase in the *Notch* signalling pathway. ADAM-17 is involved in multiple ectodomain-shedding events, most notably the release of TNF-α. 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 in vitro. ADAM-9 has been shown to promote the migration of fibroblasts *in vitro* whereas ADAM-13 expression has been detected in cranial neural-crest cells, a highly migratory population of cells in the Xenopus embryo. In support of a role for the ADAMS in implantation and placentation, ADAM-9 expression has recently been detected in the trophoblastic column, trophoblastic shell and stroma cells of the chorionic villi of the placenta of rhesus monkeys (Wang *et al.*, 2005).

1.6 ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motif)

Recent cloning studies have identified new members of the ADAM family, known as ADAMTS, in *C elegans, Drosophila* and mammals. In contrast to the ADAMS, ADAMTS are secreted proteins which do not contain the cysteine rich, EGF-like, transmembrane and cytoplasmic domains characteristic of the other members of the ADAM gene family.

1.6.1 Structural and Functional Organization of ADAMTS Subtypes

ADAMTS are characterized by four structural and functional subunits: an amino terminal prodomain, 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 (Kuno et al., 1997a; Hurskainen et al., 1999; Vazquez et al., 1999; Nath et al., 2000; Tang et al., 1999, 2001) (Figure 1). The structural features of these domains are further summarized in Table 1. Thus all numbers of this gene family have the potential to act as MMPs and to regulate cell adhesion.

Some ADAMTS subtypes have been further subclassified according to the presence of additional C-terminal modules or by the identification of common substrates. For example, ADAMTS-12 contains a mucin domain between the 3rd and 4th of its seven C-terminal TSP-1 repeats (Kuno et al. 2004). Similarly, ADAMTS-1, -4, -5, -8 and -15 have been assigned to the subfamily of ADAMTS subtypes known as the aggrecanases owing to their ability to cleave the large chondroitin sulphates: versican, brevican, aggrecan and neurocan (Tang et al., 1999, 2001; Nagase and Kashiwagi, 2003, Porter et al., 2005).

In addition to the N-terminal processing of the ADAMTS zymogens, resulting in the cleavage of the signal peptide and prodomain, C-terminal processing has been described for ADAMTS-1, -4, -8, and -12. These cleavage events occur within the spacer region and in the case of ADAMTS-12, within the mucin domain of its characteristic 2nd spacer

region (Porter et al., 2005; Tang et al., 1999). To date, one C-terminal processing event has been described for ADAMTS-1, -8 and -12, and two events for ADAMTS-4 (Kuno et al., 1997, Tang et al., 1999, 2001; Poter et al., 2005).

Removal of the ancillary C-terminal domain of the ADAMTS have a profound impact on the proetolytic activity, substrate specificity and subcellular localization of these enzymes. The C-terminal protein fragments of some of the ADAMTS also have independent and distinct biological functions. For example, the C-terminal fragment of ADAMTS-2 and -4 inhibits the enzymatic activity of the mature forms of these ADAMTS subtypes whereas those of ADAMTS-1 and -8 exhibit potent anti-angiogenic properties (Poter et al., 2005; Rodriguez-Manzaneque et al., 2000; Vazquez et al, 1999). Recently, the exogenous expression of the mature form of ADAMTS-1 was shown to have pro-metastatic effects on carcinoma cells whereas the C-terminal fragment of this ADAMTS subtype exhibited anti-metastatic effects on the cells *in vivo* (Liu et al., 2005).

The structural-functional relationship(s) and the molecular mechanisms underlying the distinct biological activities of the C-terminal fragments of these and the other ADAMTS subtypes subject to post translational cleavage remain to be elucidated.

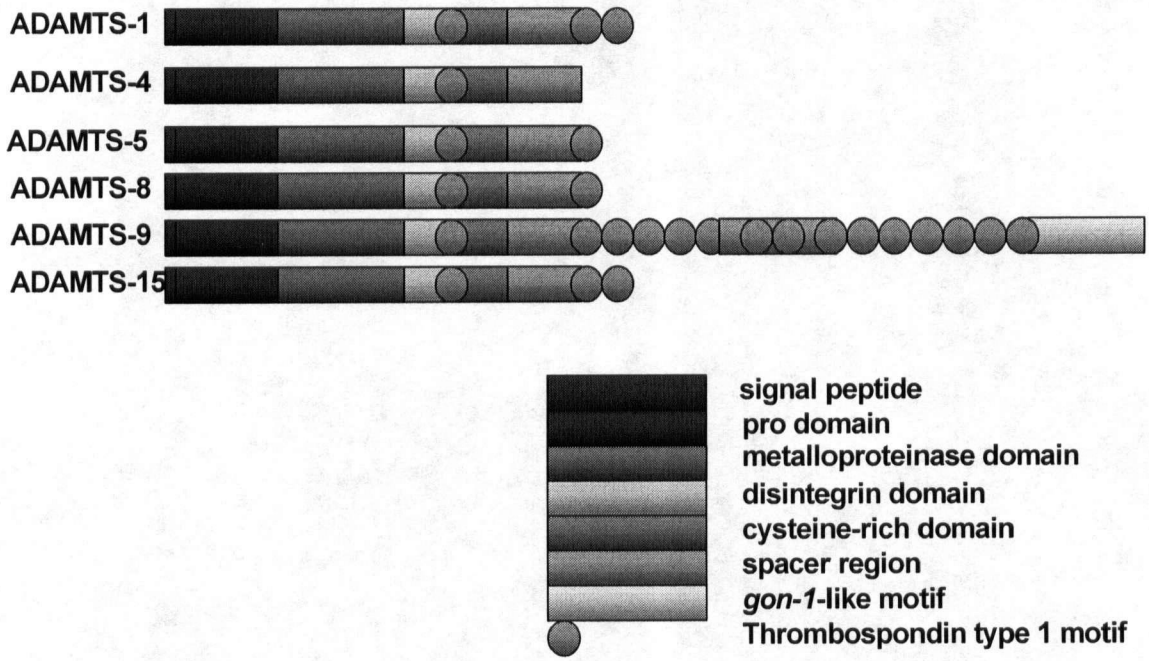


Figure 1 Diagram of ADAMTS subtypes structure. Conserved structural motifs are shown.

Table 1: Features of the structural and functional domains of the ADAMTS

DOMAIN	GENERAL FEATURES	BIOLOGICAL FUNCTIONS
Signal Peptide Prodomain	All ADAMTS contain an SPC (subtilisin-like proprotein convertase) cleavage site and with the exception of ADAMTS-10 and -12 they are all furin recognition sequences following the consensus RXR/KR. Only ADAMTS-1, -6, -7, -10, -12 and -15 contain a cysteine residue in their prodomain within a XXCGVD motif that loosely resembles that of the cysteine switch in MMPs	Responsible for maintaining latency of catalytic domain, correct protein folding and secretion. Cleaved in Golgi prior to secretion. Some ADAMTS subtypes (ADAMTS-7 and -13) can be catalytically active with their prodomains still attached.
MMP-domain	A metalloproteinase domain with a reprolysin-type zinc binding motif HEXXHXXG/N/SXXHD. The conserved aspartic acid residue (bolded) distinguishes the ADAM and ADAMTS from other MMPs and a methionine residue within the sequence V/IMAS/S or Met-turn down, downstream of the 3rd zinc binding histidine.	All ADAMTS subtypes exhibit proteolytic activity in vitro Specific substrates for most ADAMTS subtypes unidentified, however, ADAMTS-2, -13 and -14 are procollagen N-proteinases involved in processing pro-collagens to collagen. ADAMTS-1, -4, 5, -8 and -15 preferentially cleave hyalectans ADAMTS-13 cleaves the large proteoglycan, von Willebrand factor (vWF)
Disintegrin domain	Shares sequence similarity to the soluble snake venom disintegrins, a family of polypetides which conatin an integrin recognition sequence (RGD). No ADAMTS has an RGD motif in their disintegrin domain.	No biological activity identified in any ADAMTS subtype. No evidence that ADAMTS disintergin domain associates with integrins.
ECM Binding cassette • Central TSP-1 domain • Cysteine rich domain • Spacer Region • TSP-like domains	TSP-1 repeat seen in thrombospondins 1 and 2 High sequence homology among ADAMTS subtypes, contains 10 cysteine residues. Spacer region is of variable length with no distinguishing structural features. Variable number of C-terminal TSP-1 motifs (range from 0 repeats in ADAMTS-4 to 14 repeats in ADAMTS-20).	Deletion mutants demonstrated that central TSP-1, spacer region and TSP-1-like motifs all contribute to ECM binding and subsequent proteolytic activity/specificity of the ADAMTS, particularly, ADAMTS-1, -2, -4, and -8.

1.6.2 Cell Biology of ADAMTS

To date, the majority of the distinct ADAMTS subtypes have only been characterized at the structural levels. However, there is increasing evidence to suggest that these novel proteases play important roles in organogenesis during embryonic development (Cho et al.,1998; Nakamoto et al., 2005; Shindo et al., 2000), in the onset and progression of cancer (Poter et al., 2006; Held-Feindt et al., 2006), arthritis (Jones and Riley, 2005), a number of thrombotic and inflammatory conditions (Kuno et al., 1997) and in the cyclic remodeling events that occur in adult reproductive tissues (Cho et al.,1998; Boerboom et al., 2003; Young et al., 2004; Nakamoto et al., 2005).

ADAMTS-1

The best characterized member of the ADAMTS gene family is ADAMTS-1. ADAMTS-1 was initially identified as an inflammation-associated protein in an animal model for colon cancer cachexia (Kuno *et al.*, 1997a). The proteolytic activity of ADAMTS-1 has subsequently been associated with cancer (Masui *et al.*, 2001), osteoarthritis (Nagase and Kashiwagi, 2003; Jones and Riley, 2005) and in the development of inflammation associated with these two diseases (Kuno *et a*l., 1997b; Nagase and Kashiwagi, 2003) or in response to trauma (Sasaki *et al.*, 2001). For example, altered expression levels of ADAMTS-1 have been detected in human carcinomas but its individual contribution(s) to the onset and progression of cancer also remains unclear (Porter et al., 2004, 2005; Masui

et al., 2001; Rocks et al., 2006). ADAMTS-1 mRNA levels have been shown to be either increased or decreased (Porter et al., 2004) in breast carcinomas. Higher levels of this ADAMTS subtype have also being associated with pancreatic and hepatocellular cancer (Masui et al., 2004) whereas ADAMTS-1 mRNA levels are unchanged in the onset and progression of kidney cancer (Roemer et al., 2004) and decreased in lung carcinomas (Rocks et al., 2006). Among the pancreatic cancer cases, those with higher levels of ADAMTS-1 showed poorer prognosis, with evidence of increased local invasion and lymph node metastasis (Masui et al., 2004) whereas there was no direct correlation between ADAMTS-1 expression and the clinicopathological features of breast or renal carcinomas (Porter et al., 2004; Roemer et al., 2004). Furthermore, the exogenous expression of ADAMTS-1 has been shown to decrease the experimental metastasis of Chinese hamster ovary cells (Kuno et al., 2004) but increase the metastatic potential of mammary and lung cancer cell lines *in vivo* (Liu et al., 2005a). Further studies are required to evaluate the biological and clinical significance of (dys)regulated expression levels of ADAMTS-1, alone or in combination with other distinct ADAMTS subtypes, in the onset and/or progression of cancer to the later stages of the disease state.

Gene knockout studies in mice have demonstrated that ADAMTS-1 can have either redundant or non-redundant biological activities depending upon the tissue, its developmental stage or its disease state. For example, ADAMTS-1 has redundant roles in the growth and development of cartilage and bone (Little et al., 2005), in cartilage degradation during the progression of arthritis (Stanton et al., 2005) but has non-

redundant roles in follicular development and ovulation (Shindo et al., 2000; Shozu et al., 2005).

ADAMTS-1 and the Endometrium

Studies from our laboratory have recently determined that ADAMTS-1 is also spatiotemporally expressed in the human endometrium during the menstrual cycle and in pregnancy (Ng et al., 2006). In particular, ADAMTS-1 expression was readily detectable throughout the glandular epithelium but was restricted to the predecidualised stromal cells surrounding the spiral arterioles of the secretory endometrium. Extensive ADAMTS-1 immunostaining was subsequently detected in the stromal cells of first trimester decidua, large polyhedral cells that are characteristic of this dynamic tissue. This expression pattern suggests that ADAMTS-1, mediates at least in part, the steroid-mediated ECM remodeling events that occur in endometrium during each menstrual cycle.

ADAMTS-1 expression has also been detected in the uterine tissues of pregnant mice (Shindo et al., 2000; Mittaz et al., 2004) but the role of this novel metalloproteinase in the development of a uterine environment that is capable of supporting pregnancy remains unclear. Although there is a significant increase in ADAMTS-1 mRNA levels in the mouse endometrium during the peri-implantation period (Kim et al., 2005), endometrial tissues of mice null-mutant for this gene have been shown to either develop large cysts (Shindo et al., 2000) or be capable of undergoing normal morphological decidualisation (Mittaz et al., 2004). However, all ADAMTS-1 gene knockout female mice are reported

to have reduced pregnancy rates (Shindo et al., 2000; Mittaz et al., 2004).

1.7 HYPOTHESIS

To date, the factors responsible for the spatiotemporal expression of ADAMTS-1 in the endometrium have not been identified. However, as ADAMTS-1 expression is associated with the decidualisation of endometrial stromal cells of both murine and human endometrium, a developmental process that is governed by increasing levels of P4 and a concomitant decrease in E2, we hypothesis that gonadal steroids are potent regulators of endometrial ADAMTS-1 expression levels. In support of this hypothesis, P4 has recently been shown to regulate ADAMTS-1 expression in rodent and porcine ovaris (Doyle et al., 2004; Shimada et al., 2004).

Specific Aim 1: To examine the ability of progestins, estrogens and androgens to regulate ADAMTS-1 mRNA and protein expression levels in primary cultures of human endometrial stromal cells.

To determine the regulatory effects of gonadal steroids on ADAMTS-1 expression in the human endometrium, we will examine the abilities of P4, E2, and DHT, alone or in combination, to regulate ADAMTS-1 mRNA and protein expression levels in a dose- and time-dependent manner. This will be achieved by using primary cultures of human endometrial stromal cells. The ability of these gonadal steroids to regulate ADAMTS-1

mRNA and protein levels in these primary cell cultures will be determined by using a real-time PCR strategy and Western blot analysis, respectively.

Specific Aim 2: To examine the regulatory effects of antisteroidal compounds on ADAMTS-1 mRNA and protein expression levels in primary cultures of human endometrial stromal cells.

We will next examine the regulatory effects of the antisteroidal compounds, RU486 (an anti-progestin), ICI 181, 872 (an anti-estrogen) or flutamide (an anti-androgen) alone or in combination with P4, E2, and DHT, alone or in combination, to regulate ADAMTS-1 mRNA and protein expression levels in a dose- and time-dependent manner. This will be achieved by using the same primary cell cultures and approaches as described above.

PART 2 MATERIALS AND METHODS

2.1 Tissues

Endometrial tissues were obtained from women of reproductive age undergoing hysterectomy for reasons other than endometrial cancer. All patients had normal menstrual cycles and had not received hormones for at least 3 months prior to tissues collection.

2.2 Cell Isolation and Culture

Enriched cultures of stromal cells were isolated from these endometrial tissues by enzymatic digestion and mechanical dissociation as previously described (Chen et al .)

In this protocol, endometrial tissue samples are minced and subjected to 0.1% collagenase (type IV, sigma Chemical Co, St Lois, MO) and 0.1% hyaluronidase (type I-S, sigma Chemical Co, St Lois, MO) digestion in a shaking water bath at 37°C for 60 min. The cell digest are then passed through a nylon sieve (38μm). The isolated glands and any undigested tissue fragments are retained on the sieve, and the eluate containing the stromal cells was collected in a 50ml tube. The stromal cells are then pelleted by centrifugation at 800 × g for 10 min at room temperature. The cell pellet are washed once with phenol red-free DMEM containing 10% charcoal-stripped fetal bovine serum (FBS) before being resuspended and plated in phenol red-free DMEM containing 25 mM

glucose, L-glutamine, antibiotics (100U/ml penicillin, 100μg/ml streptomycin) and supplemented with 10% charcoal-stripped FBS, The culture medium is replaced 30 min after plating to reduce epithelial cell contamination. The purity of the endometrial stromal cell cultures is determined by immunocytochemical staining for vimentin (fibroblast), cytokeratin (epithelial), muscle actin (muscle cells) and factor VIII (endothelial). These cellular markers have been used to determine the purity of human endometrial cell cultures (Irwin *et al.*, 1989). As defined by these criteria, the endometrial stromal cell cultures used in these studies contained < 1% epithelial or vascular cells.

2.3 Experimental Culture Conditions

2.3.1 Steroid Treatments

Endometrial stromal cells (passage 2) were grown to confluence, washed with PBS and cultured in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS and containing either increasing concentrations of P4 (1-10μM), E2 (1-100nM), or DHT (1-200nM) for 72h or a fixed concentrations of P4 (1μM), E2 (30nM) or DHT (100nM) for 0, 6, 12, 24, 48, or 72h.

To determine whether a combination of steroids was required for maximal ADAMTS-1 expression in endometrial stromal cells, the cells were cultured in the presence of P4 plus E2, P4 plus DHT or E2 plus DHT for 0-72 h before being harvested for real-time PCR and Western blot analysis.

2.3.2 Antisteroid Treatments

Endometrial stromal cells were cultured in the presence or absence of increasing concentrations of RU486 (25nM, 250nM, 2.5μM, 5μM, and 10μM), ICI 182, 780 (10nM, 100nM, 1μM, 5μM, 10μM)) or flutamide (1nM, 10nM, 100nM, 500nM, 1μM) for 72 hours, or fixed concentration of RU486 (2.5μM), ICI 182, 780 (1μM) or flutamide (100nM) for 0, 6, 12, 24, 48 or 72 hours.

Endometrial stromal cells cultured with vehicle (0.1% ethanol) served as controls for these studies. The concentrations of gonadal steroids and antisteroidal compounds and the time points examined in this study based upon previous studies (Chen et al, 1998, 1999; Ling et al., 2002). All of the primary cultures of endometrial stromal cells were harvested for either total RNA or protein extraction.

2.4 RNA Preparation and Synthesis of First Strand cDNA

Total RNA was prepared from endometrial tissue cell cultures using Tri-Reagent (Bio/Can, Mississauga, Canada) and protocol recommended by the manufacturer. The total RNA extracts were then treated with Deoxyribonuclease-1 (Sigma Aldrich) to eliminate possible contamination with genomic DNA. To verify the integrity of the RNA, aliquots of the total RNA was electrophoresed in a 1 % (w/v) denaturing agarose gel containing 3.7% (v/v) formaldehyde and the 28 S and 18 S ribosomal RNA subunits

visualized by ethidium bromide staining. The purity and 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, Mississuaga, ON, Canada)

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

2.5 Design of Oligonucleotide Primers

Nucleotide sequences specific for human ADAMTS-1 were identified in the Genebank database using the BLAST (basic Local Alignment Search Tool) computer program (www.ncbi.com). Forward and reverse oligonucleotide primers specific for ADAMTS-1 or GAPDH, which served as an internal control for these studies, were designed by the PRIMER EXPRESS software (Applied Biosystems). The specific nucleotide sequences of these primers are listed in Table 2.

Table 2 Primer sequences for real-time PCR analysis

Gene	Primer sequence(5'-3')	Position	Product(bp)
GAPDH Forward	ATGGAAATCCCATCACCATCTT	269-290	57
GAPDH Reverse	CGCCCCACTTGATTTTGG	325-308	
ADAMTS-1 Forward	GCTCATCTGCCAAGCCAAAG	1979-1999	59
ADAMTS-1 Reverse	CTACAACCTTGGGCTGCAAAA	2037-2016	

2.6 Real-time PCR Analysis

Real-time PCR was used to detect ADAMTS-1 mRNA levels in our primary cultures of human endometrial stromal cells. GAPDH mRNA levels served as endogenous control for these studies. Real-time PCR was performed using the ABI PRISM 7000 sequence detection system and SYBR green master mix reagent (Applied Biosystems).

The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta C}{}_T$ method. For the treated samples, evaluation of $2^{-\Delta\Delta C}{}_T$ indicates the fold change in gene expression relative to the control. $\Delta C_T = C_{T.Target} - C_{T.GAPDH}$. $\Delta\Delta C_T = (C_{T.Target} - C_{T.GAPDH})_X - (C_{T.Target} - C_{T.GAPDH})_0$. The C_T value represents the cycle number at which a fluorescent signal rises statistically above background. X is any time or dose point of treated sample, 0 represented control sample (Kenneth and Thomas 2001). Data were analyzed using SDS 2.0 software (Applied Biosysterms).

2.7 Western Blot Analysis

Endometrial stromal cell cultures were washed three times in cold 1% PBS and incubated in 100 µl of cell extraction buffer (Biosource International, Camarillo, CA) supplemented with 1.0 mM PMSF and protease-inhibitor cocktail at 4°C for 30 minutes on a rocking platform. The cell lysates were centrifuged at 10, 000 x g for 20 minutes at 4°C and the supernatants will be used for Western blot analysis. The concentrations of protein in the cell lysates were determined using a BCA kit (Pierce Chemicals, Rockford, IL). Using a polyclonal antibody directed against human ADAMTS-1 (Biodesign Intl., Saco, ME). To standardize the amounts of protein loaded into each lane, the blots were reprobed with a monoclonal antibody directed against human β-actin (Sigma Chemical Co.). The Amersham ECL system was used to detect the amount of each antibody bound to antigen and the resultant autoradiograms analyzed by UV densitometry. The absorbance values obtained for ADAMTS-1 were then normalized relative to the corresponding β-actin absorbance value.

2.8 Statistical Analysis

The absorbance values obtained from the real-time PCR products and the autoradiograms generated by Western blotting were subjected to statistical analysis using GraphPad Prism 4 computer software (GraphPad, San Diego, CA). Statistical differences between the absorbance values were assessed by the analysis of variance (ANOVA). Differences

were considered significant for $p \leq 0.05$. Significant differences between the means were determined using Dunnett's test. The results are presented as the mean relative absorbance ($\pm$ SEM) obtained using cell cultures isolated from tissue samples obtained from $\geq$3 different patients.

PART 3 RESULTS

3.1 Time-Dependent Effects of Gonadal Steroids on Stromal ADAMTS-1 mRNA and Protein Levels

ADAMTS-1 mRNA was detected in all of the endometrial stromal cell cultures. The addition of vehicle to the culture medium had no significant effect on the levels of the ADAMTS-1 mRNA transcript present in these cells at any of the time points examined in these studies (data not shown).

A significant increase in ADAMTS-1 mRNA levels was detected in endometrial stromal cells cultured in the presence of either P4 or DHT after 24h (Figure 2 A and B, respectively). ADAMTS-1 mRNA levels in these cells continued to increase until the termination of these studies at 72h. In contrast, E2 alone did not significantly increase stromal ADAMTS-1 mRNA levels, at least at any of the time points examined in these studies (Figure 2 C).

Western blot analysis revealed the presence of a single ADAMTS-1 protein species of 110 kDa in all of the endometrial stromal cell cultures. This protein species corresponds to the ADAMTS-1 zymogen (Rodriguez-Manzaneque et al., 2000; Wachsmuth et al., 2004). In agreement with our PCR data, ADAMTS-1 protein expression levels remained relatively constant in endometrial stromal cells cultured in the presence of vehicle alone (data not shown). Similarly, P4 and DHT but not E2 caused a significant increase in

ADAMTS-1 protein expression levels in endometrial stromal cells over time in culture (Figure 3A-C, respectively).

3.2 Dose-Dependent Effects of Gonadal Steroids on Stromal ADAMTS-1 mRNA and Protein Levels

A significant increase in ADAMTS-1 mRNA and protein expression levels was only observed in cells cultured with the higher concentrations of P4 (1μM or 5μM; Figure 4A and 5A) or DHT (100nM or 200nM; 4B and 5B) examined in these studies. In contrast, ADAMTS-1 mRNA and protein expression levels remained relatively constant in cells cultured in the presence of increasing concentrations of E2 (Figure 4 C and 5C).

3.3 Combinatorial Effects of Gonadal Steroids on Stromal ADAMTS-1 mRNA and Protein Expression Levels

P4 plus DHT caused a significant increase in stromal ADAMTS-1 mRNA levels over time in culture (Figure 6) that were not significantly different from those observed in endometrial stromal cells cultured in the presence of P4 or DHT alone. In contrast, the levels of the ADAMTS-1 protein species present in these cell cultures were significantly greater than those detected in cells cultured in either gonadal steroid over the same time periods (Figure 6).

In contrast to endometrial stromal cells cultured in the presence of either P4 or DHT alone, we failed to detect any significant changes in ADAMTS-1 mRNA and protein expression levels in cells cultured in E2 plus P4 (Figure 7) or E2 plus DHT (Figure 8) at any of the time points examined in these studies.

3.4 Effects of Varying Concentrations of E2 to Attenuate the P4-mediated Increase in Stroma ADAMTS-1 mRNA and Protein Levels

In agreement with our preceding findings, P4 increased ADAMTS-1 mRNA and protein levels after 72 h of culture (Figure 9). There was no significant difference between ADAMTS-1 mRNA and protein levels in these cells and those cultured with the lower concentrations of E2 (0.1nM and 1nM) examined in these studies. However, a significant decrease in ADAMTS-1 mRNA and protein levels was first detected in cells cultured in the presence of 10 nM of E2. The addition of higher concentrations (30nM and 100nM) of E2 to the culture medium did not result in a further decrease in ADAMTS-1 mRNA and protein levels in these cells.

3.5 Regulatory Effects of the Antisteroidal Compounds, RU486, ICI 182, 780 and Flutamide on Stromal ADAMTS-1 mRNA and Protein Expression Levels

ADAMTS-1 mRNA and protein expression levels remained relatively constant in endometrial stromal cells cultured in the presence of RU486 at least at the concentrations of this antisteroidal compound examined in our studies (Figure 10).

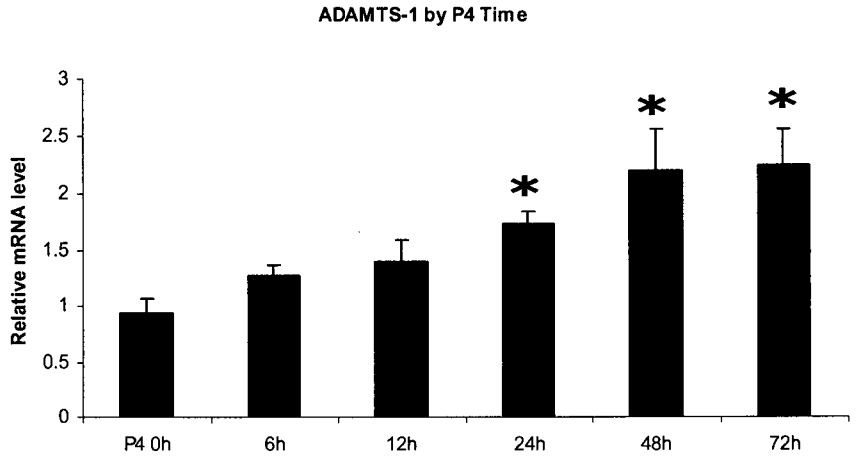
RU 486 inhibited the P4-mediated increase in stromal ADAMTS-1 mRNA and protein expression levels in a concentration-dependent manner (Figure 11). In contrast, ADAMTS-1 mRNA and protein expression levels remained elevated in endometrial stromal cells cultured with a combination of DHT and increasing concentrations of RU 486 (Figure 12).

Similarly, there was no significant difference in ADAMTS-1 mRNA and protein expression levels in endometrial stromal cells cultured with increasing concentrations of hydroxyflutamide alone (Figure 13). Hydroxyflutamide inhibited the DHT-mediated increase in stromal ADAMTS-1 mRNA and protein expression levels in a concentration-dependent manner (Figure 14).

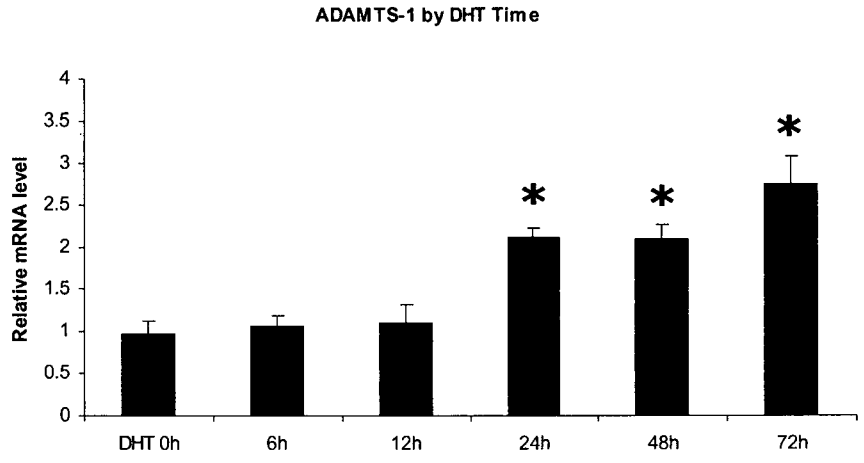
Increasing concentrations of ICI 182, 780, alone or in combination with E2, had no significant effect on stromal ADAMTS-1 mRNA and protein expression at least at the concentrations we studied (Figure 15, 16).

Flutamide has no significant effect on ADAMTS-1 mRNA and protein levels expression (Figure 15A and B). Flutamide significant inhibited the DHT-mediated increase in ADAMTS-1 at mRNA and protein levels (Figure 16A and B).

2A



2B



2C

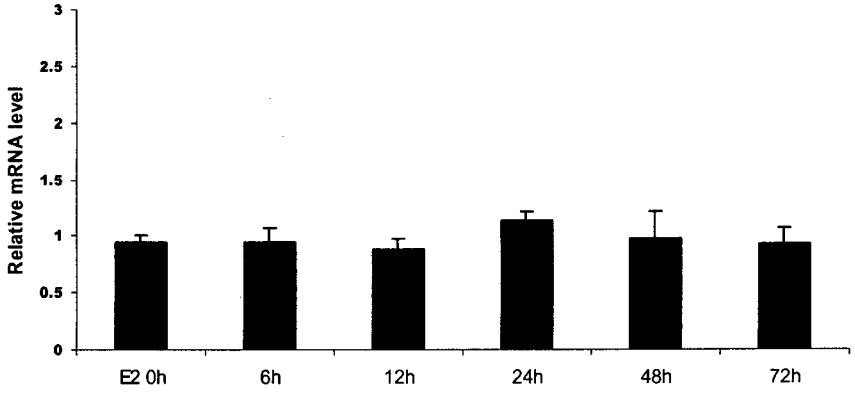
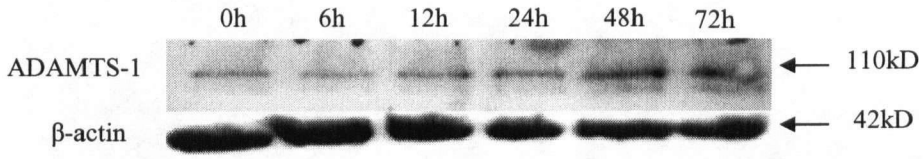
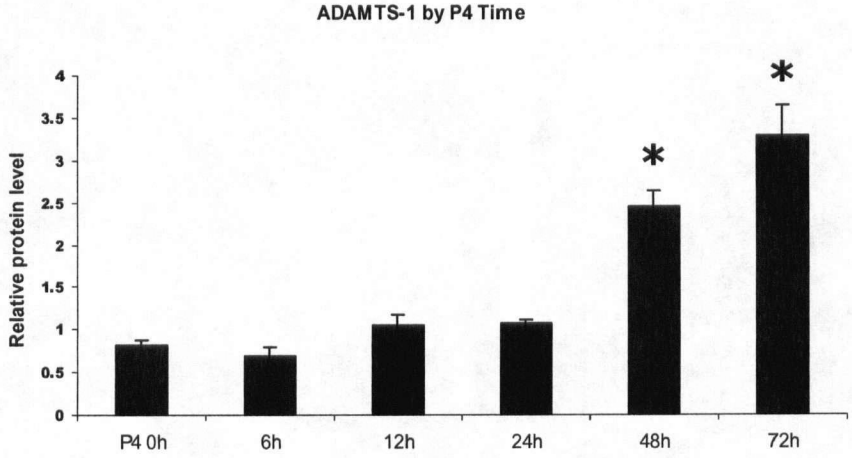


Figure 2

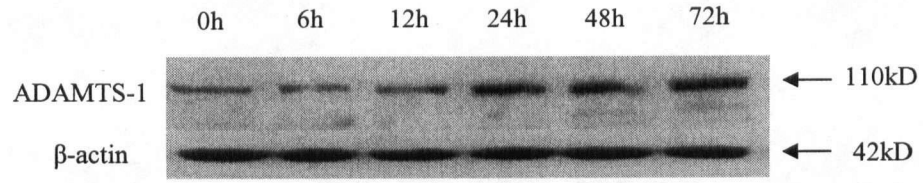
Figure2. Time-dependent effects of gonadal steroids on ADAMTS-1 mRNA levels in endometrial stromal cells. Real-time PCR analysis of ADAMTS-1 mRNA levels in endometrial stroma cells cultured in the presence of a fixed concentration of (A) P4 (1μM), (B) DHT (100nM) or (C) E2 (30nM) for 0, 6, 12, 24, or 48 h. The results are presented as mean+SEM, n>3 in the bar graphs (* $P<0.05$ vs. untreated control).

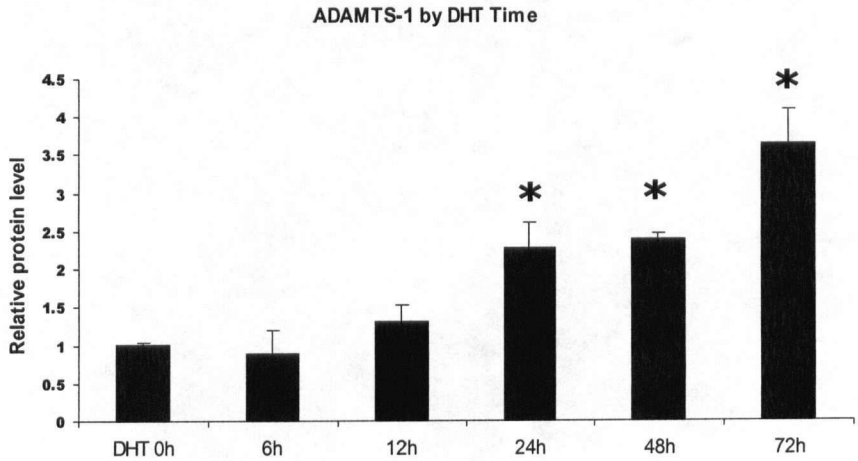
3A


0h
6h
12h
24h
48h
72h
ADAMTS-1
β-actin
110kD
42kD


ADAMTS-1 by P4 Time
Relative protein level
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
P4 0h
6h
12h
24h
48h
72h

3B


0h
6h
12h
24h
48h
72h
ADAMTS-1
β-actin
110kD
42kD


ADAMTS-1 by DHT Time
Relative protein level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
DHT 0h
6h
12h
24h
48h
72h

3C

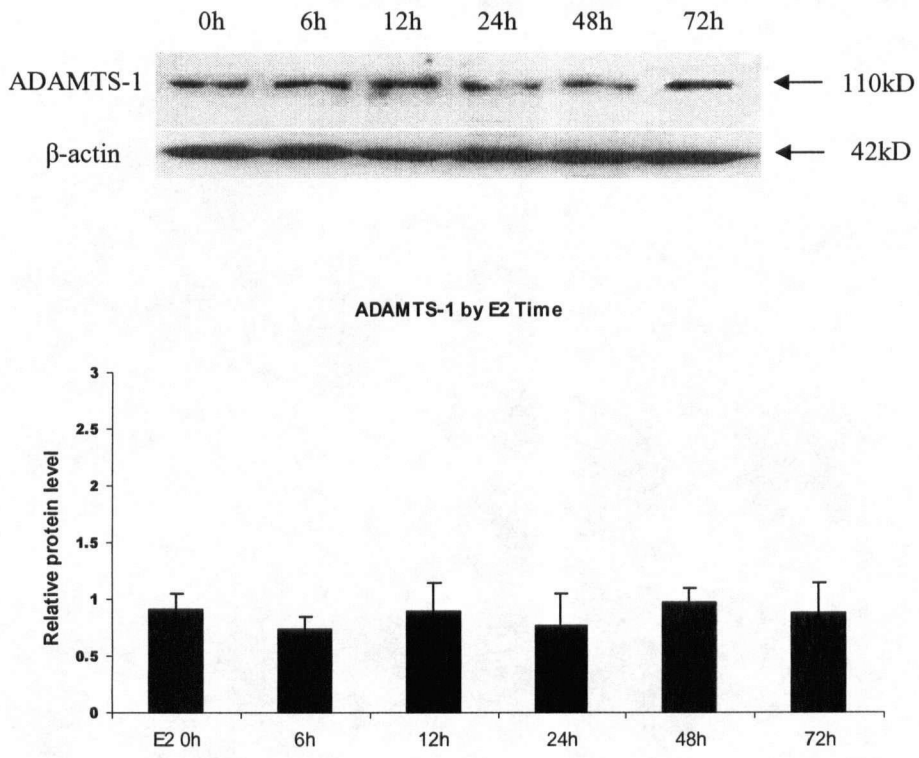
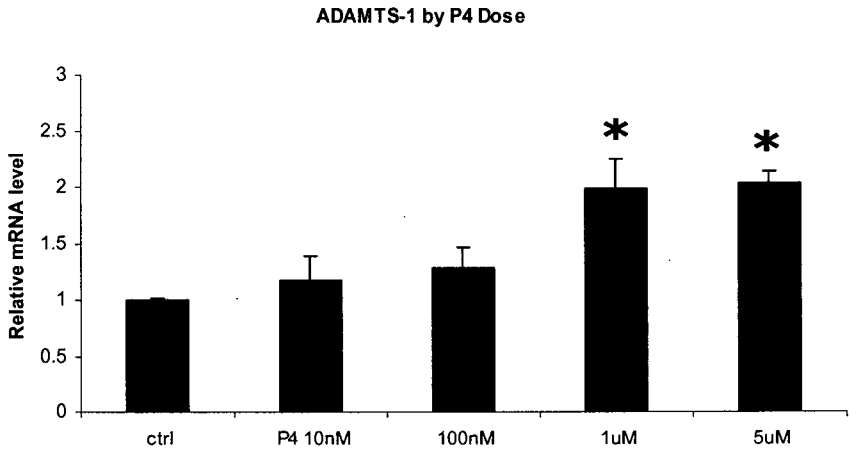


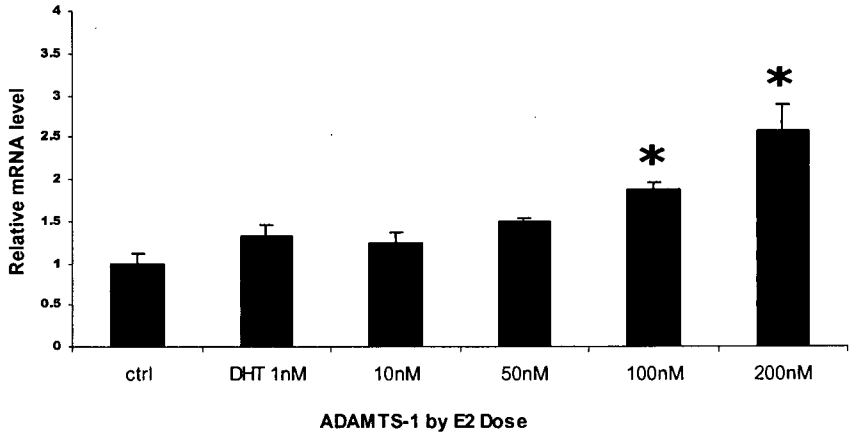
Figure 3

Figure 3. Time-dependent effects of gonadal steroids on ADAMTS-1 protein expression levels in endometrial stromal cells. Western blot analysis was performed using total protein extracts (30μg) prepared from endometrial stromal cells cultured in the presence of a fixed concentration of (A) P4 (1μM), (B) DHT (100nM) or (C) E2 (30nM) for 0, 6, 12, 24, or 48 hours. The blots were probed with a polyclonal antibody directed against ADAMTS-1 or a mononoclona. The Amersham ECL system was used to detect antibody bound to antigen. The resultant autoradiograms were scanned and the values obtained for ADAMTS-1 normalized to absorbance values obtained for the corresponding b-actin. The results derived from this analysis, as well as those from at least three other studies (autoradiograms not shown) were standardized to the 0h control and are represented (mean + SEM; $n > 3$) in the bar graphs (* $P<0.05$ vs. untreated control).

4A



4B



4C

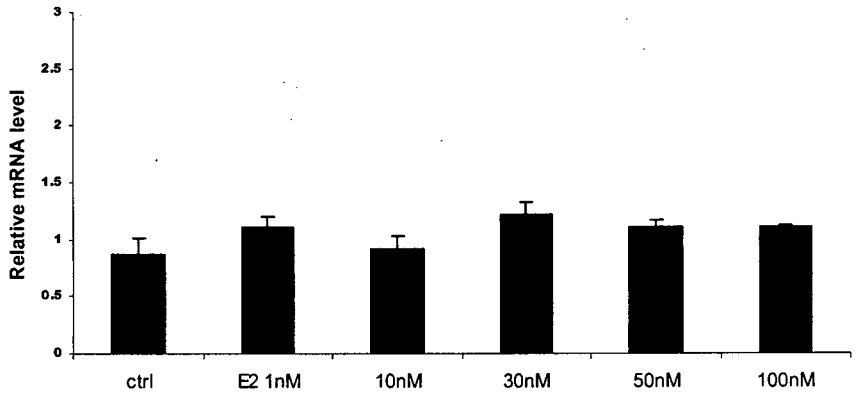


Figure 4

Figure 4. ADAMTS-1 mRNA and protein levels in endometrial stromal cells cultured in the presence of increasing concentration of P4, DHT, or E2 for 72 hours. Results are presented as Mean±SEM, n≥3 in the bar graphs, (* $P<0.05$ vs. untreated control). (A) Cells cultured with 0, 10nM, 100nM, 1μM, 5μM, 10μM of P4 for 72 hours. (B) Cells cultured with 0, 1nM, 10nM, 50nM, 100nM, 200nM of DHT for 72 hours. (C) Cells cultured with 0, 1nM, 10nM, 30nM, 50nM, 100nM of E2 for 72 hours.

5A

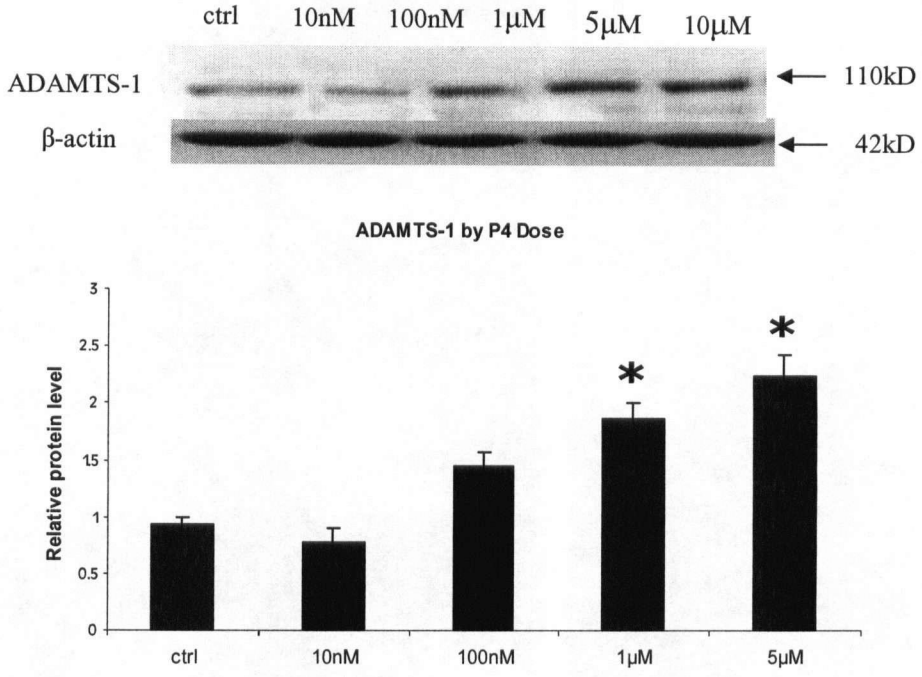
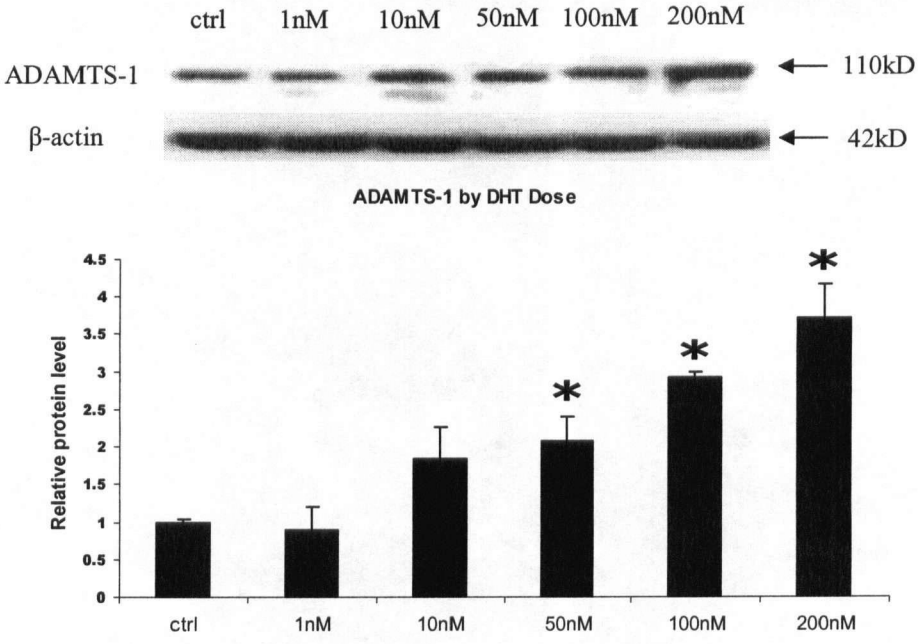


Figure 3D

5B



5C

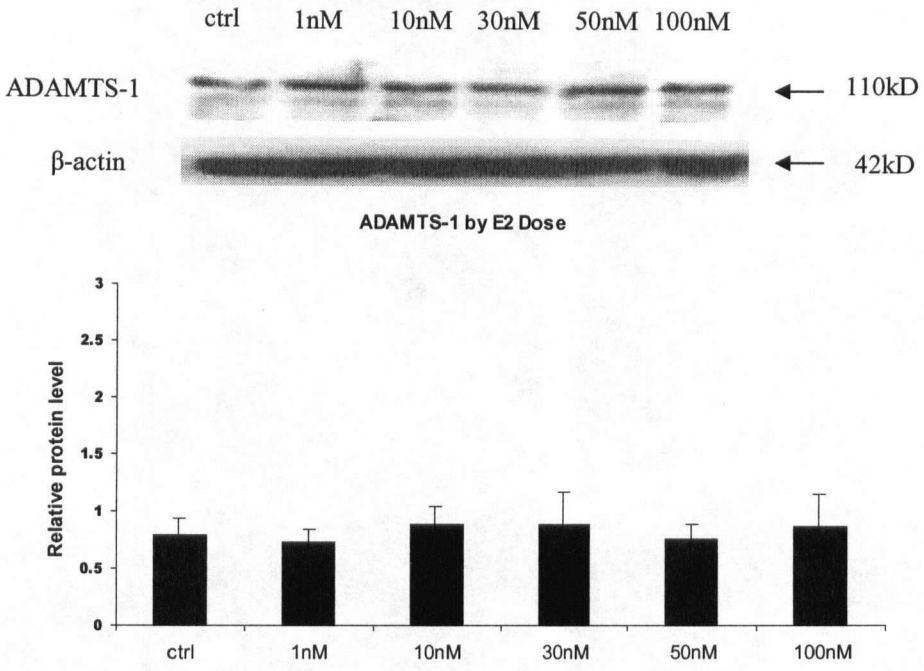
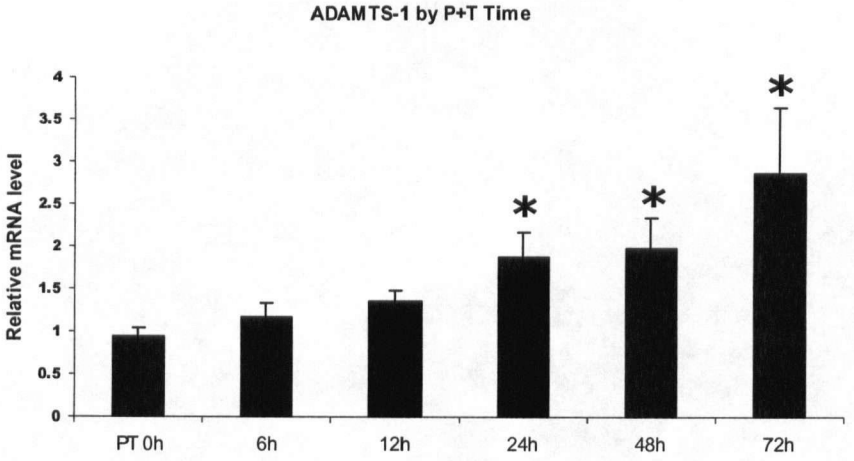


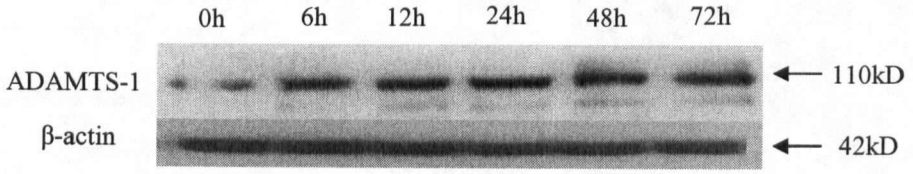
Figure 5

Figure 5. ADAMTS-1 mRNA and protein levels in endometrial stromal cells cultured in the presence of increasing concentration of P4, DHT, or E2 for 72 hours. Panel D-F, Western blot analysis of ADAMTS-1 expression in protein extraction (30μg) prepared from endometrial stromal cells cultured in the presence of increased concentration of P4 (0-10μM), DHT (0-200nM) and E2 (0-100nM) for 72 hours respectively. Result presented as Mean±SEM, $n \geq 3$ in the bar graphs (* $P<0.05$ vs. untreated control).

6A



6B



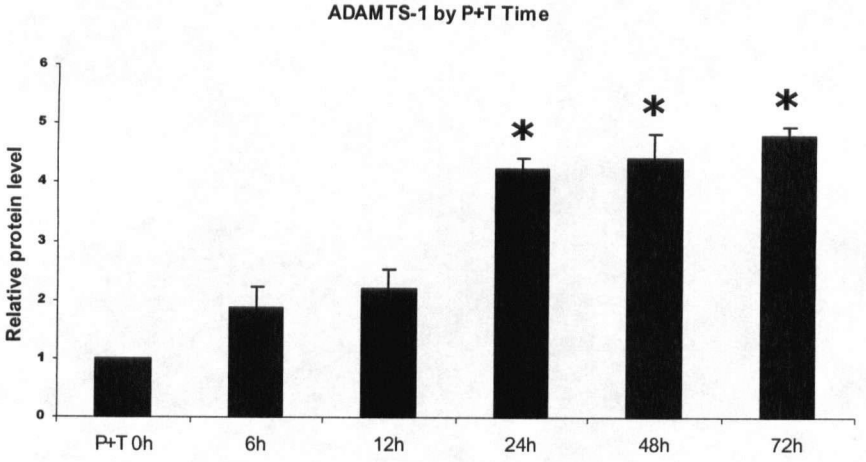
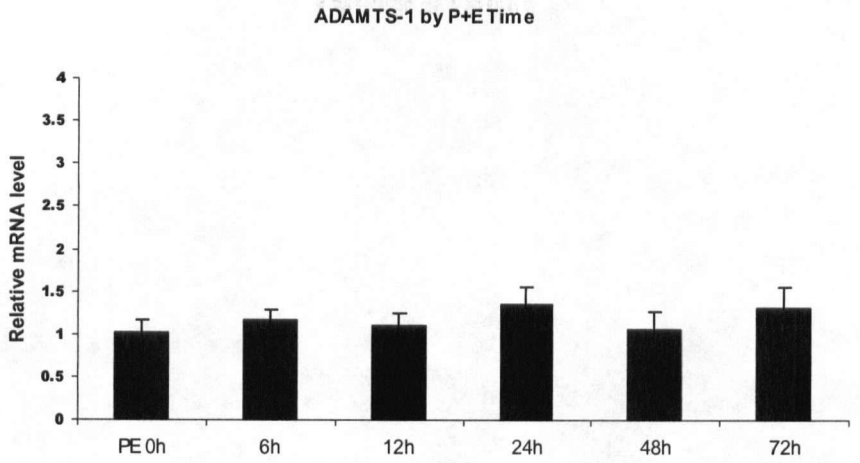


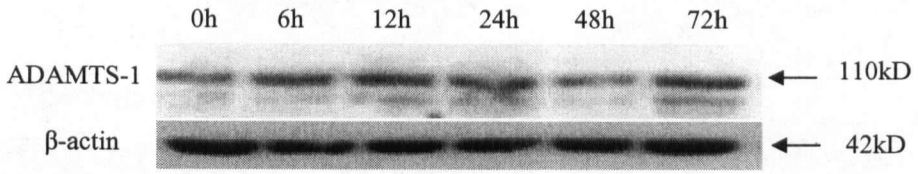
Figure 6

Figure 6. Expression of combinatorial effects of gonadal steroids on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Representative ADAMTS-1 levels in cells cultured in the presence of P4 (1μM) plus DHT (100nM) for 0, 6, 12, 24, 48, and 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs* (* $P<0.05$ *vs.* untreated control). (A) Real-time PCR analysis of ADAMTS-1 mRNA levels. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

7A



7B



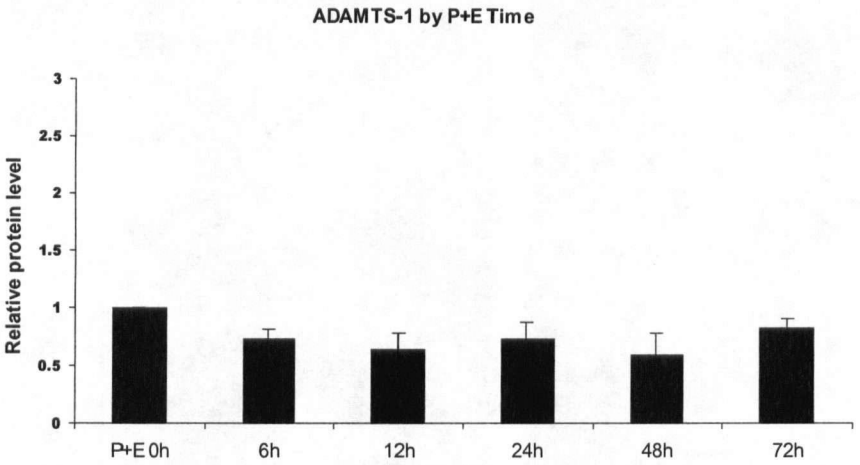
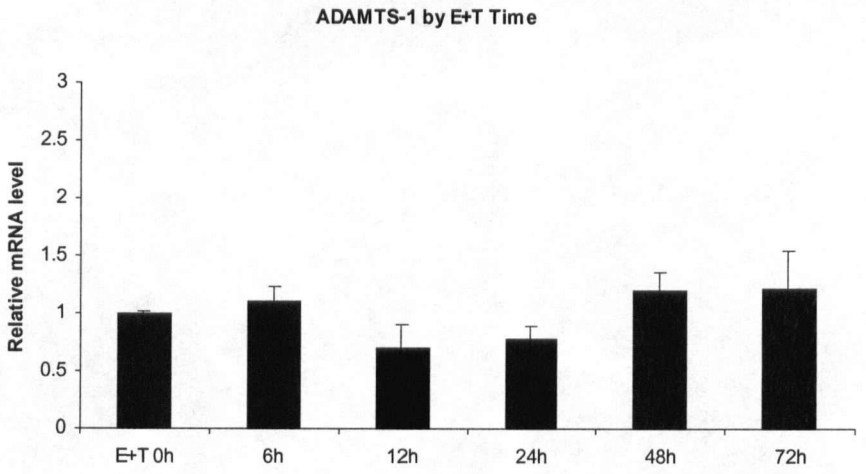


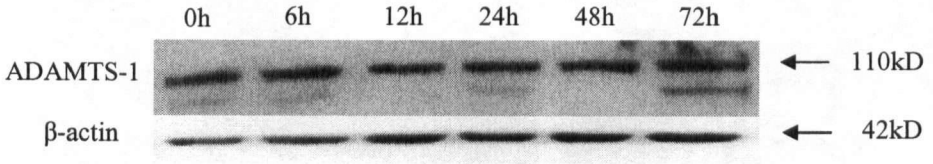
Figure 7

Figure 7. Expression of combinatorial effects of gonadal steroids on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Representative ADAMTS-1 levels in cells cultured in the presence of P4 (1μM) plus E2 (30nM) for 0, 6, 12, 24, 48, and 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*.(A)Real-time PCR analysis of ADAMTS-1 mRNA levels. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

8A



8B



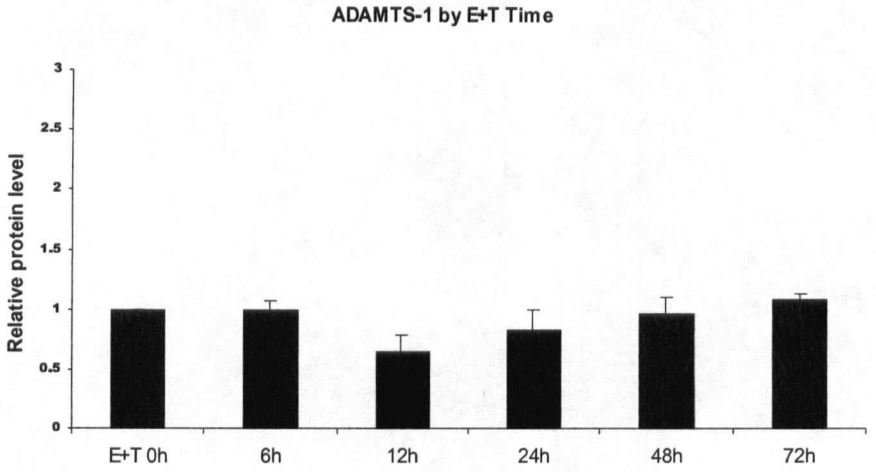
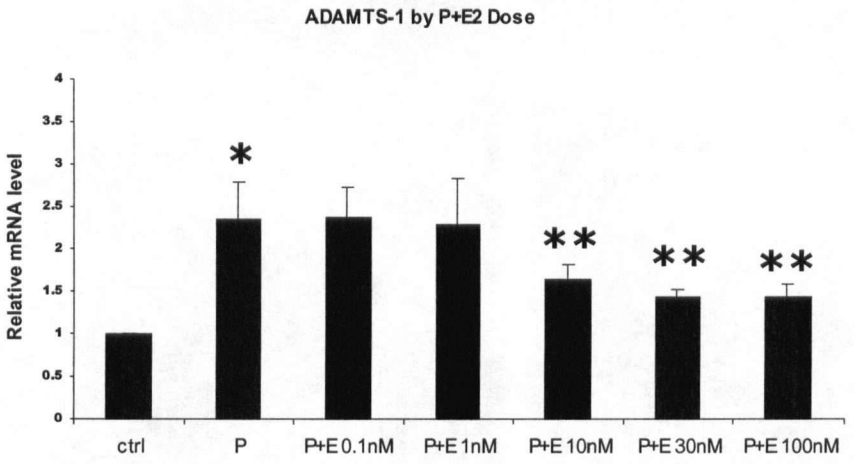


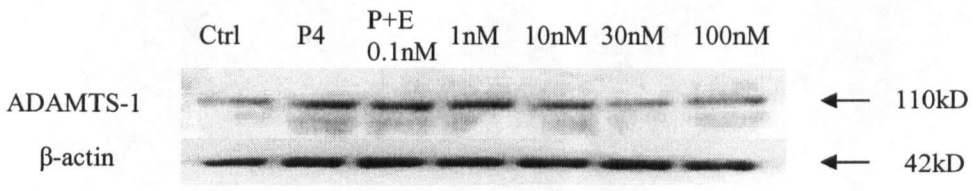
Figure 8

Figure 8. Expression of combinatorial effects of gonadal steroids on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Representative ADAMTS-1 levels in cells cultured in the presence of DHT (100nM) plus E2 (30nM) for 0, 6, 12, 24, 48, and 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*. (A) Real-time PCR analysis of ADAMTS-1 mRNA levels. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

9A



9B



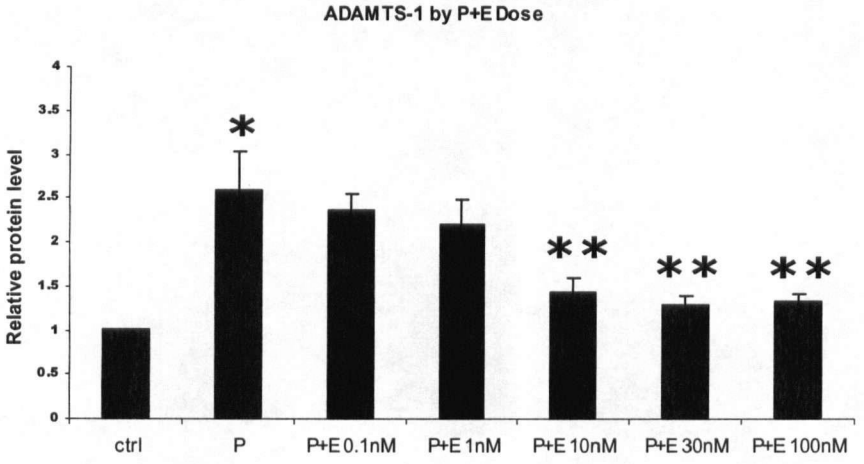
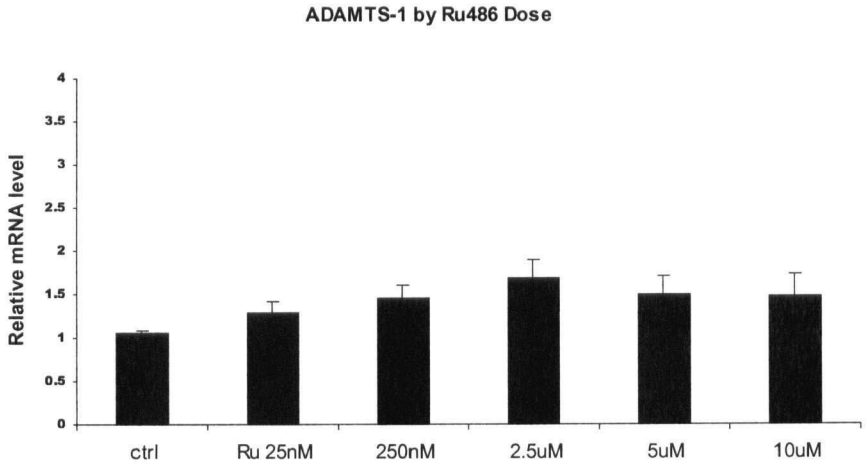


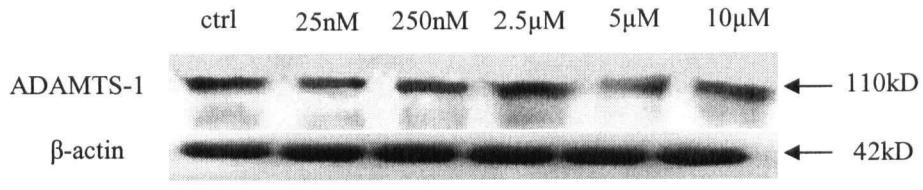
Figure 9

Figure 9. Expression of ADAMTS-1 mRNA and protein levels in endometrial stromal cells cultured in the presence of P4 (1μM) plus increased concentration of E2 (0.1nM, 1nM, 10nM, 30nM, 100nM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs* (* P<0.05 *vs.* untreated control, ** P<0.05 *vs.* P4). (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

10A



10B



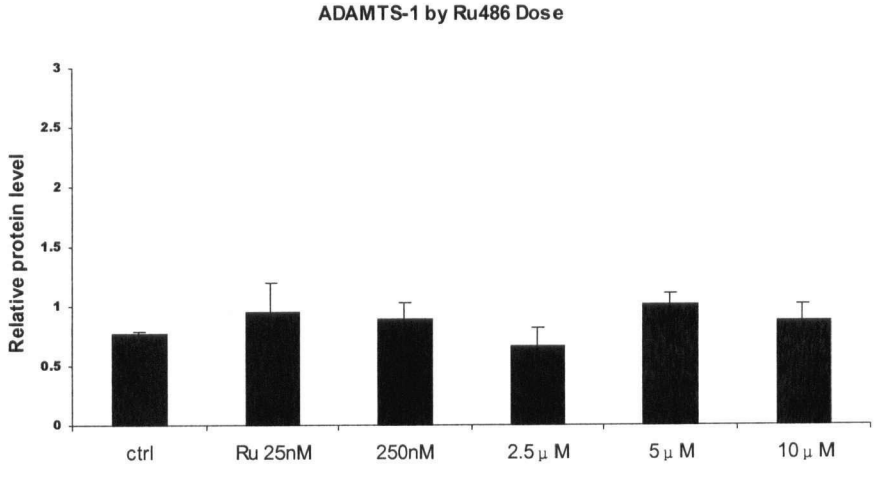
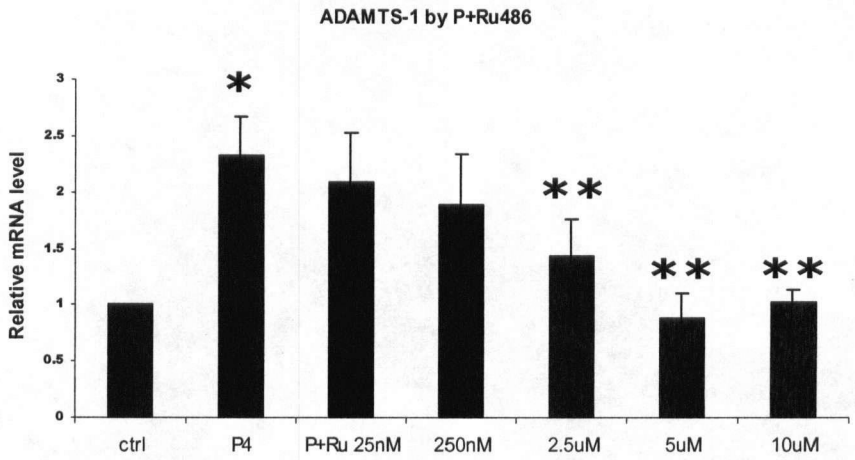


Figure 10

Figure 10. Representative regulatory effects of antiprogestin compound RU486 on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Panel A and B, cell cultured in the presence of 0, 25nM, 250nM, 2.5μM, 5μM, 10μM of RU486 for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*. (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

11A



11B

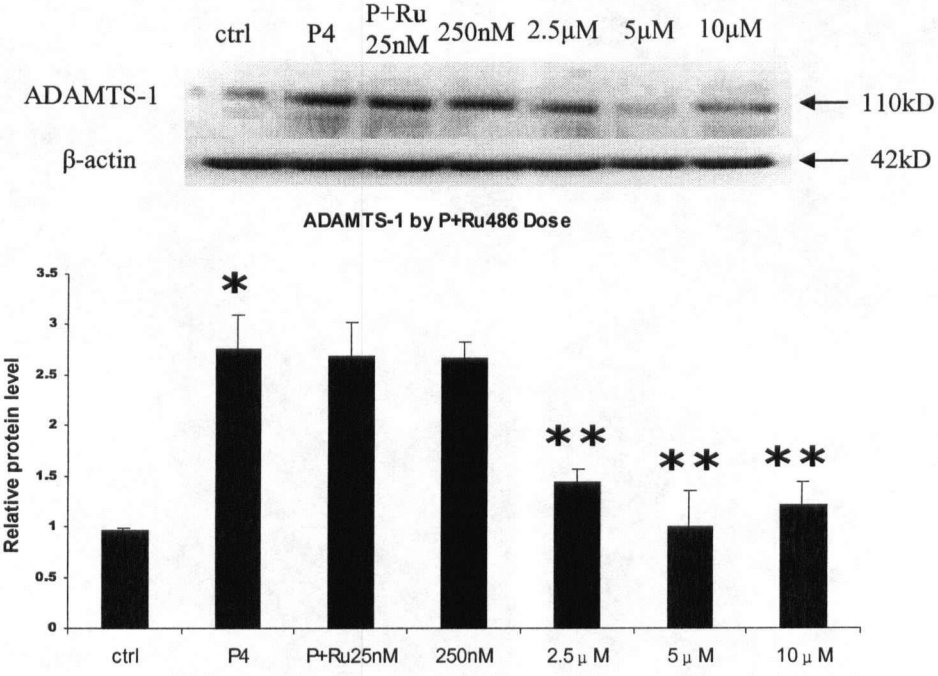
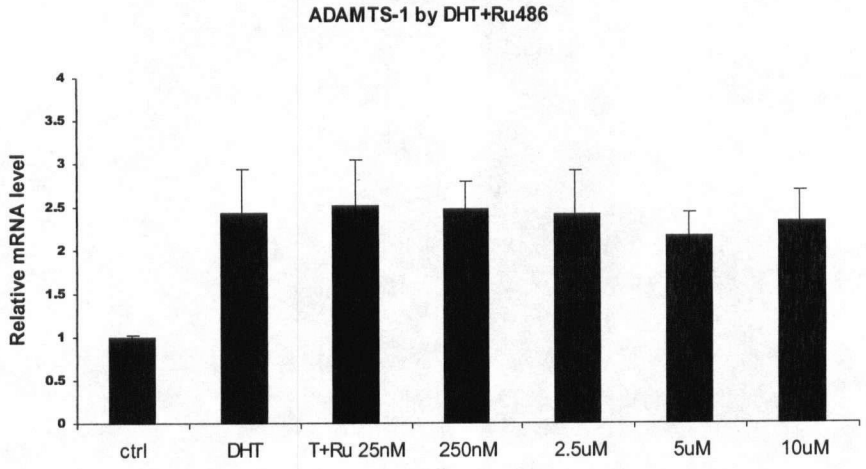


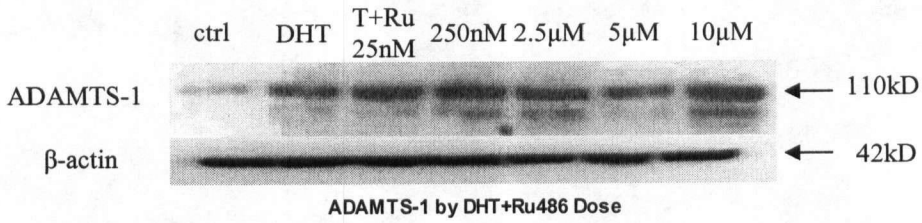
Figure 11

Figure 11. Representative regulatory effects of antiprogestin compound RU486 on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Expression of ADAMTS-1 levels in endometrial stromal cells cultured in the presence of P4 (1μM) plus increased concentration of RU486 (25nM, 250nM, 2.5μM, 5μM, 10μM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs* (* $P<0.05$ *vs.* untreated control, ** $P<0.05$ *vs.* P4). (A) Real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

12A



12B



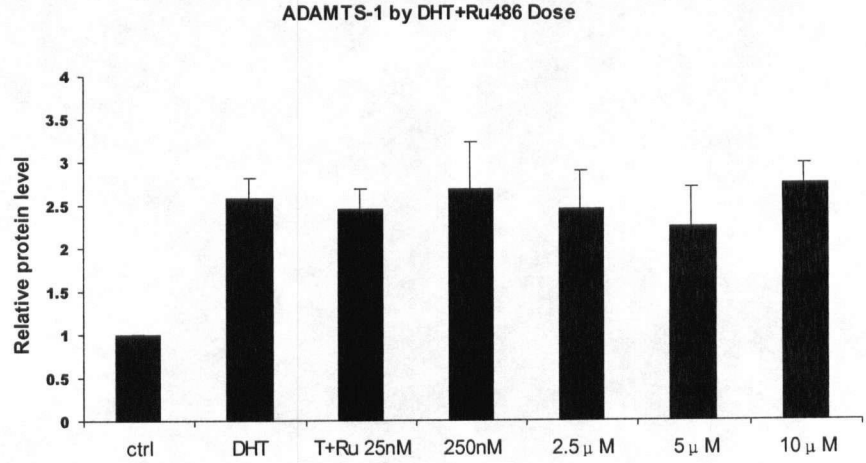
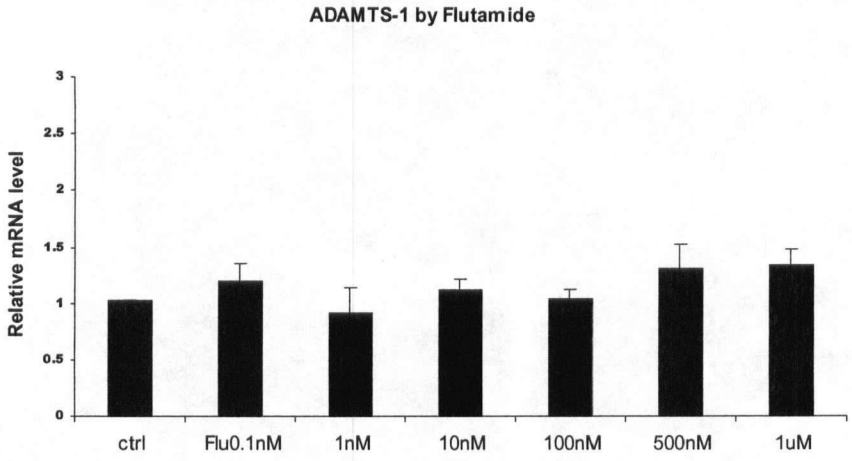


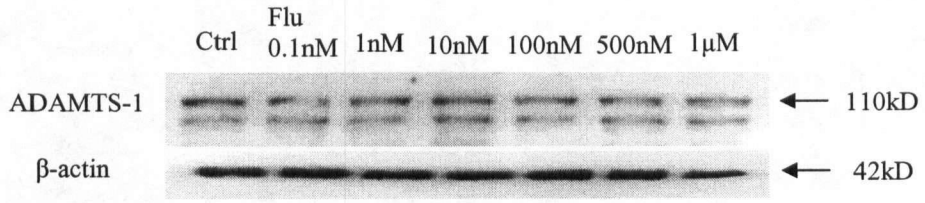
Figure 12

Figure 12. Representative regulatory effects of antiprogesterone compound RU486 on ADAMTS-1 mRNA and protein levels in endometrial stromal cells. Panel E and F, Expression of ADAMTS-1 levels in endometrial stromal cells cultured in the presence of DHT (100nM) plus increased concentration of RU486 (25nM, 250nM, 2.5μM, 5μM, 10μM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*. (A) Real-time analysis of ADAMTS-1 mRNA levels. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

13A



13B



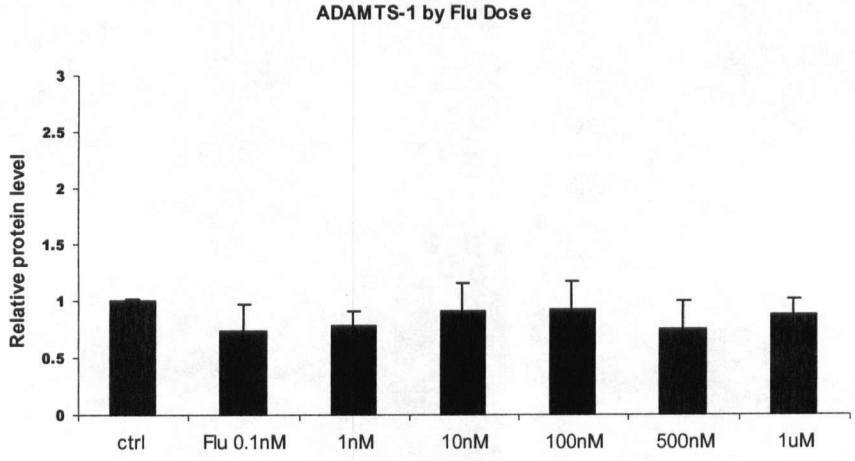
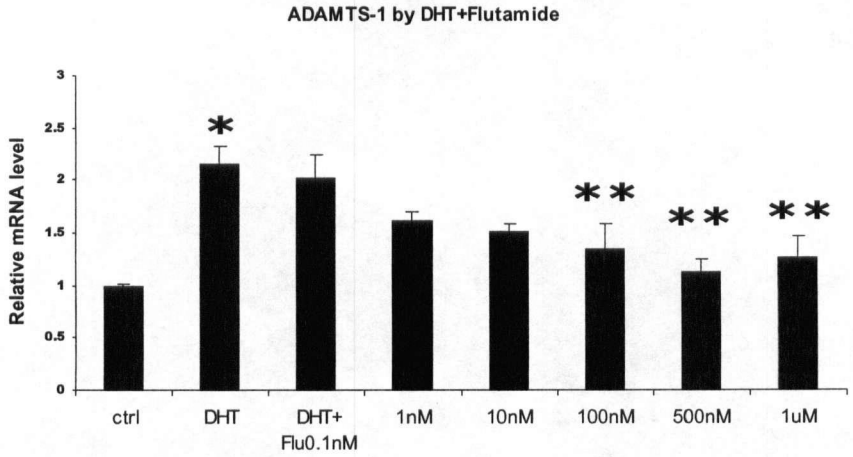


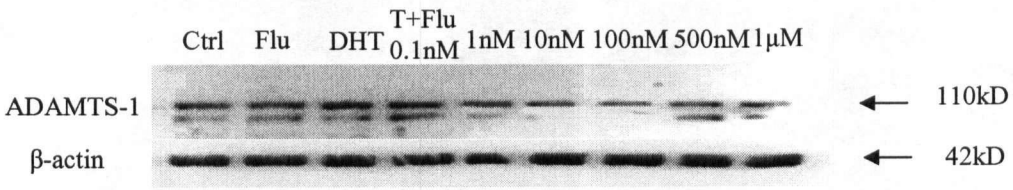
Figure 13

Figure 13. Expression of ADAMTS-1 mRNA levels in endometrial stromal cells cultured in the presence of increased concentration of flutamide (1nM, 10nM, 100nM, 500nM and 1μM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs.* (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

14A



14B



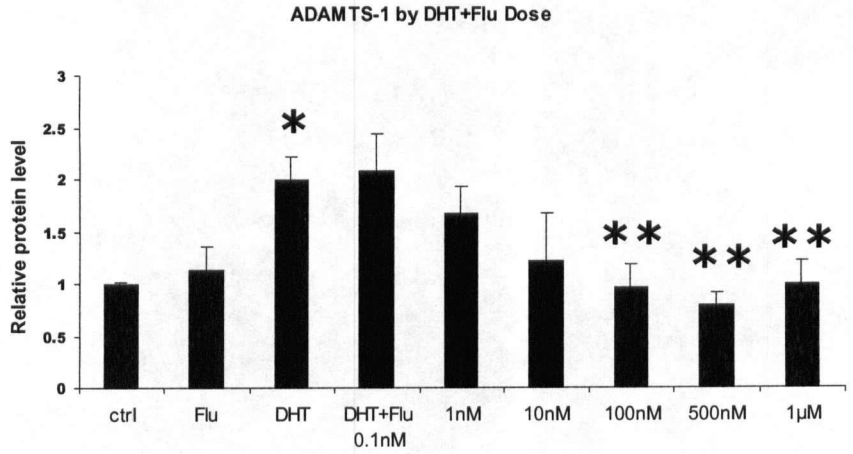
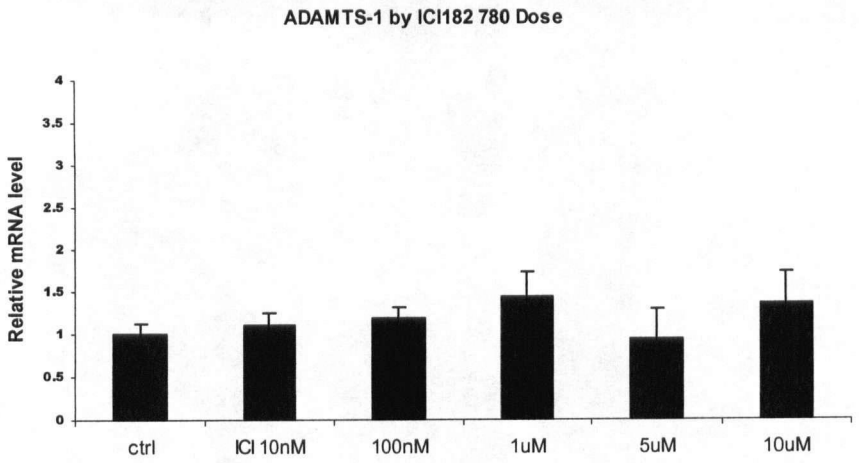


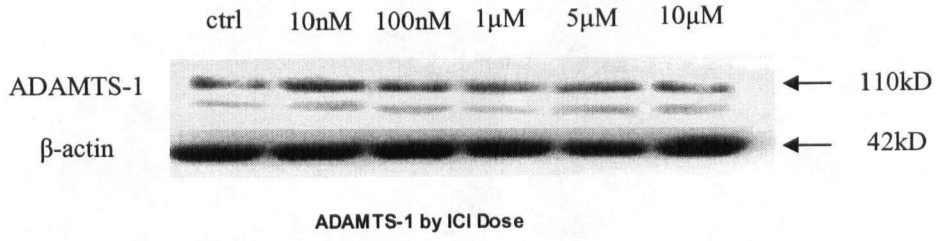
Figure 134

Figure 14. Expression of ADAMTS-1 mRNA levels in endometrial stromal cells cultured in the presence of DHT (100nM) plus increased concentration of flutamide (1nM, 10nM, 100nM, 500nM 1μM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*. (* $P<0.05$ *vs.* untreated control, ** $P<0.05$ *vs.* DHT). (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

15A



15B



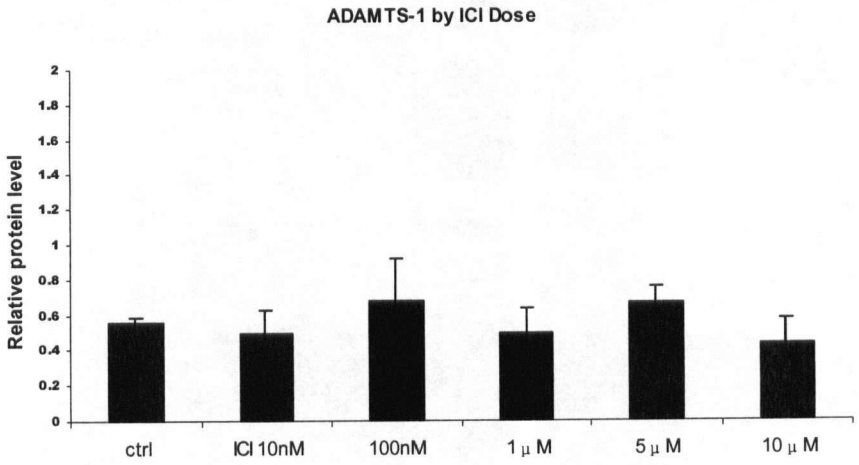
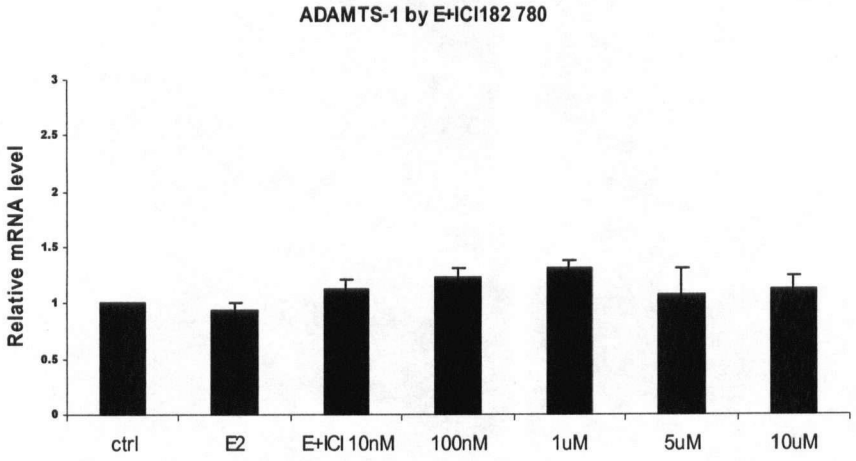


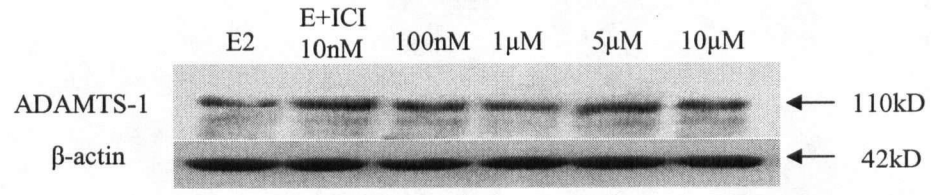
Figure 145

Figure 15. Expression of ADAMTS-1 mRNA levels in endometrial stromal cells cultured in the presence of 0, 10nM, 100nM, 1μM, 5μM, 10μM of ICI 182 780 for 72 hours, Results are presented as Mean±SEM, n≥3 in the *bar graphs.* (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

16A



16B



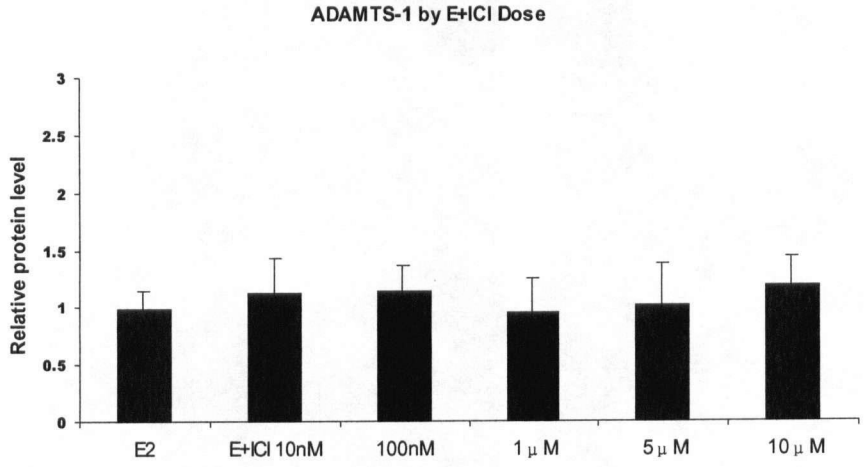


Figure 156

Figure 16. Expression of ADAMTS-1 mRNA levels in endometrial stromal cells cultured in the presence of E2 (30nM) plus increased concentration of ICI 182, 780 (10nM, 100nM, 1μM, 5μM, 10μM) for 72 hours. Results are presented as Mean±SEM, n≥3 in the *bar graphs*. (A) Representative real-time analysis of ADAMTS-1 mRNA level. (B) Western blot analysis of ADAMTS-1 expression in protein extracts (30μg) prepared from the endometrial stromal cells.

PART 4 DISCUSSION

In these studies, I have determined that that the regulation of ADAMTS-1 mRNA and protein expression levels in human endometrial stromal cells by gonadal steroids involves a complex interplay between progestins, estrogens and androgens. In particular, P4 and DHT increased ADAMTS-1 expression levels whereas E2 alone had no regulatory effect on the expression levels of this ADAMTS subtype in these primary cell cultures. A combination of DHT and P4 potentiated the increase in the levels of the ADAMTS-1 protein species present in these cell cultures whereas E2 was capable of attenuating the stimulatory effects of both P4 and DHT on stromal ADAMTS-1 mRNA and protein expression levels. In contrast, RU486 and hydroxyflutamide specifically inhibited the increase in ADAMTS-1 expression levels mediated by P4 and DHT, respectively.

ADAMTS-1 mRNA transcripts have been detected in a wide array of adult human tissues including term placenta and non-pregnant uterus (Abbaszade et al., 1999; Vazquez et al., 1999). ADAMTS-1 expression has also been detected in the uterine tissues of pregnant mice (Shindo et al., 2000; Mittaz et al., 2004) but the role of this novel metalloproteinase in the development of a uterine environment that is capable of supporting pregnancy remains unclear. Although there is a significant increase in ADAMTS-1 mRNA levels in the mouse endometrium during the peri-implantation period (Kim et al., 2005), endometrial tissues of mice null-mutant for this gene have been shown to either develop large cysts (Shindo et al., 2000) or be capable of undergoing normal morphological decidualisation (Mittaz et al., 2004). However, all ADAMTS-1 gene knockout female

mice are reported to have reduced pregnancy rates (Shindo et al., 2000; Mittaz et al., 2004). Taken together, these observations suggest that ADAMTS-1 is neither necessary nor sufficient to mediate decidualisation but may play an important role in the later stages of implantation and placentation and/or that other ADAMTS subtypes expressed in the endometrium may have overlapping and thus, non-redundant functions in this multi-step reproductive process. A potential candidate for the partial rescue of the reproductive capacity of the ADAMTS-1 gene knockout mouse is ADAMTS-5, also known as aggrecanase-2, ADAMTS-11 or by its trivial name "implantin" (Hurskainen et al., 1999). Both proteinases are expressed in the mouse decidua (Hurskainen et al., 1999; Shindo et al., 2000; Mittaz et al., 2004) and preferentially cleave members of the gene family of chondroitin sulphate glycoproteins known as the hyalectins (Apte, 2004; Porter et al., 2005). Furthermore, mice null-mutant for the ADAMTS-5 gene are viable and fertile (Stanton et al., 2005). To our knowledge, any reciprocal and compensatory changes in the expression levels of distinct ADAMTS subtypes in the endometrium of either ADAMTS-1 or ADAMTS-5 gene knockout mice have not been examined.

ADAMTS-1 is a secreted, multidomain, multifunctional protein composed of an amino terminal prodomain, a proteolytic 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 3 TSP-like motifs) (Kaushal and Shah, 2000; Tang, 2001; Apte, 2004; Porter et al., 2005). In addition to its proteolytic activity, ADAMTS-1 has been shown to have both angioinhibitory and angiogenic properties *in vitro* and *in vivo* (Vazquez et al., 1999; Carpizo and Iruela-Arispe, 2000; Shindo et al., 2000). Thus, ADAMTS-1 has the

potential to contribute to the development of an uterine environment capable of supporting a pregnancy via the regulated degradation of the endometrial ECM and/or the extensive vascular changes that occur in this dynamic tissue during the menstrual cycle, another steroid-mediated developmental process implantation.

Initial biochemical studies predicted that the ADAMTS-1 zymogen (110 kDa) undergoes two consecutive post-translational cleavage steps that generate two distinct bioactive fragments (87 kDa and 67 kDa) of this protein (Rodriguez-Manzaneque et al., 2000). All three of the ADAMTS-1 protein species have subsequently been detected in cellular extracts prepared from mouse ovaries (Russell et al., 2003). However, similar to our findings, only the ADAMTS-1 zymogen was detected in primary cultures of human chondrocytes (Wachsmuth et al., 2004). These differences are likely attributable to the loss of endogenous proteolytic factors capable of cleaving ADAMTS-1 (Rodriguez-Manzaneque et al., 2000) following the isolation and culture of these enriched populations of cells. Of these proteolytic factors, only MMP-2 has been detected in the human endometrium (Fata et al., 2000). However, MMP-2 activity in primary cultures of endometrial stromal cells is dependent upon the presence of soluble factor(s) derived from the glandular epithelium (Goffin et al., 2002).

P4 increased ADAMTS-1 mRNA and protein expression in our cultures of endometrial stromal cells in a concentration and time-dependent manner. Similarly, P4 has been shown to be a key regulator of ADAMTS-1 in the rodent ovary. The ability of the progestin synthesis epostane, to inhibit the preovulatory increase in ADAMTS-1 mRNA

levels in rat follicles, which was also not observed in mice null-mutant for the progesterone receptor provided indirect evidence that P4 is a key regulator of ADAMTS-1 in the ovary. However, the levels of the mRNA transcript encoding this ADAMTS subtype were found to be higher in early stage corpus luteum when P4 levels are low and decline during the mid luteal phase of the estrous cycle when the circulating levels of this gonadal steroid are high, suggesting that other factors are involved in the regulation of ADAMTS-1 mRNA levels, at least in the ovary (Madan et al., 2003). Computer-based searches of the nucleotide sequence and functional assays have subsequently failed to identify a PR response element in the promoter region of the murine ADAMTS-1 gene (Doyle et al., 2004). Instead, P4 appears to regulate ADAMTS-1 gene expression, at least in the mouse, through an indirect mechanism(s) that involves the DNA binding transcription factors Sp1/Sp3, C/EBPb and/or NF-1 (Doyle et al., 2004). Interestingly, interleukin-1β (IL-1β) and tansforming growth factor- β1 (TGF-β1), two cytokines which have been shown to regulate ADAMTS-1 in several mammalian cell types including decidual stromal cells (Ng et al., 2006), mediate many of the biological actions of P4 on the human endometrium (Graham and Lala, 1992; Godkin and Dore, 1998; Salamonsen et al., 2000, 2003; Fazleabas et al., 2004) and to regulate gene expression in other human cell types via the Sp1/Sp3 complex (Chadjichristos et al., 2002, 2003).

E2 alone did not alter ADAMTS-1 mRNA or protein expression levels in isolated endometrial stromal cells. In agreement with these findings, ADAMTS-1 expression is low in proliferative endometrium of mice and humans and in preovulatory follicles of rodent, bovine and equine preovulatory follicles when E2 is the predominant steroid.

Similarly, previous studies have failed to demonstrate a direct effect of E2 on the expression of several markers of decidualisation including intgerin subunits and PRL and IGFBP-1 (Osteen et al., 1989) or the secretion of MMPs (Osteen et al., 1994) by endometrial stromal cells *in vitro*. Furthermore, although E2 is essential for the regulated expression of a myriad of proteins in the endometrium (Nantermet et al., 2005), depleted levels of this gonadal steroid during the luteal phase of the menstrual cycle do not appear to adversely affect endometrial development *in vivo* (Younis et al., 1994). The ability of mice null mutant for ER to undergo decidualisation provides further evidence that E2 is not required for the activation of signaling pathways that control this developmental process via, at least in part increased ADAMTS-1 expression. Our studies indicate that E2 has a suppressive effect on the ability of P4 and DHT to increase ADAMTS-1 expression levels in endometrial stromal cells in vitro. Similarly, there is a marked increase in ovarian levels of ADAMTS-1 following the gonadotropin surge when there is an increase in P4 levels and a concomitant decrease in estrogen levels decline in follicular fluid (Boerboom et al., 2003). To date, the molecular mechanisms by which E2 modulates the regulatory effects of P4 and DHT remain to be elucidated but are unlikely to involve alterations in the expression of PR and/or AR in these cells.

The relative potencies of P4 and DHT on ADAMTS-1 expression levels were similar, with a combination of both gonadal steroids producing an additive stimulatory effect. Taken together, these observations suggest that DHT has a specific effect that is mediated by a pathway that is cooperative but independent from that of P4 ADAMTS-1. Similar results have been observed with the biological actions of P4 and DHT on decidualisation

of the rodent endometrium. DHT and other androgens have been shown to have a direct effect on the human endometrium and mimic the biological actions of P4 *in vitro* and *in vivo*. For example, both progesterone and androgens antagonize the E2-meditaed proliferation of endometrial cells *in vivo*. Furthermore, T and DHT can substitute for P4 in inducing secretion of PRL and IGFBP-1, two biochemical markers of decidualisation, in primary cultures of human endometrial stromal cells *in vitro* and in maintaining the decidualisation of the mouse endometrium *in vivo* (Zhang and Croy, 1996). The possibility that ARs mediate DHT actions is supported by the ability of flutamide/hydroxyflutamide to block the regulatory effects of this non-aromatisable androgen on stromal ADAMTS-1 expression levels. Hydroxyflutamide has also been shown to be suppressive of the decidual cell reaction in the rodent endometrium (Chandrasekhar et al., 1991, Zhang and Croy, 1996). However, unlike the regulatory effects of DHT in human endometrial stromal cells, RU486 was capable of partially inhibiting the biological actions of DHT on the rodent endometrium suggesting that it is mediated, at least in part by its cross-reactivity with PR. In contrast to our findings, hydroxyflutamide had no significant effect on ADAMTS-1 expression levels in porcine cumulus-oocyte complexes suggesting that the ability of androgens to regulate this ADAMTS subtype may be dependent upon the cellular context.

In summary, my studies demonstrated the regulation of ADAMTS-1 in human endometrial stromal cells *in vitro* involves a complex interplay between progestins, estrogens and androgens.

PART 5 CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

5.1 Conclusion

In conclusion, I have determined that P4 and DHT regulate ADAMTS-1 mRNA and protein expression levels in primary cultures of endometrial stromal cells in a concentration and time-dependent manner. In contrast, E2 does not have this regulatory effect on stromal ADAMTS-1 expression levels. Although these observations are indicative of the expression pattern of previously described for ADAMTS-1 in the human endometrium during the menstrual cycle, care should be taken in extrapolating our findings to the *in vivo* situation. Elucidation of the molecular mechanisms by which these gondal steroids regulate ADAMTS-1 expression in endometrial stromal cells and the biological function(s) of this ADAMTS subtype in the human endometrium will require further experimentation. However, my findings provide a basis for future studies into the expression and function(s) of ADAMTS-1 in the human endometrium under normal and pathological conditions.

5.2 Limitations

These *in vitro* studies my not directly correlated to *in vivo* situation. The biological function of ADAMTS-1 in endometrium remains to be elucidated.

5.3 Future direction

1. To investigate ADAMTS-1 expression in endometrium under pathological conditions, such as endometrial cancer, infertility,and endometrosis.
2. To examin the molecular mechanisms underlying steroids regulate of ADAMTS-1 in endometrial stromal cells.

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