A BIOLOGICAL ROLE FOR GnRH I AND GnRH II
AT THE MATERNAL-FETAL INTERFACE

BY

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M.D, CHINA MEDICAL COLLEGE, TAIWAN, 1985

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PROGRAM OF REPRODUCTIVE AND DEVELOPMENTAL SCIENCES

WE ACCEPT THIS THESIS AS CONFORMING TO THE REQUIRED STANDARD.

THE UNIVERSITY OF BRITISH COLUMBIA
JULY 2003
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ABSTRACT

Remodeling of the endometrial extracellular matrix, which occurs during the early stages of pregnancy in the human, is mediated by the temporal expression of urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMP) in both the maternal and fetal compartments and counterbalanced by their respective endogenous inhibitors, plasminogen activator inhibitor (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs) in both an autocrine and paracrine manner. To date, the factors capable of regulating the expression of uPA and MMP proteolytic systems at the maternal-fetal interface remain poorly characterized. However, the direct correlation between the expression of Gonadotropin-Releasing Hormone (GnRH I) and the activity of uPA, MMP-2 and MMP-9 in the human endometrium and placenta has led to the hypothesis that GnRH I may play a regulator of this developmental event. In these studies, we have examined the ability of GnRH I and the second mammalian form of GnRH (GnRH II), which is also expressed in the human endometrium and placenta, to regulate uPA/PAI-1 and MMP-2, MMP-9/TIMP-1 mRNA and protein expression levels in primary cultures of decidual stromal cells or extravillous cytotrophoblasts isolated from first trimester tissues. GnRH I and GnRH II increased uPA, MMP-2 and MMP-9 mRNA and protein expression levels in both cell types. In contrast, GnRH I and GnRH II were capable of increasing TIMP-1 levels in extravillous cytotrophoblasts but had no significant effect on its expression levels in decidual stromal cell cultures. Furthermore, both forms of GnRH down-regulated PAI-1 mRNA levels in extravillous cytotrophoblasts whereas GnRH I increased and GnRH II decreased PAI-1 mRNA and protein expression levels in these decidual cell cultures. Antagonists specific for the
GnRH I receptor, Cetrorelix and Antide, were capable of inhibiting the regulatory effects of GnRH I, but not GnRH II on these primary cell cultures. Collectively, these observations strengthen our hypothesis that GnRH is a key regulator of the proteolytic degradation of the endometrial ECM during implantation. However, the observed biological actions of GnRH I and GnRH II on these primary cell cultures appear to be mediated by distinct, tissue-specific molecular mechanisms, which as yet, remain to be elucidated.
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<tr>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<td>Diacylglycerol</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<td>g (as in xg)</td>
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<td>GDP</td>
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<td>Guanosine triphosphate</td>
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<td>HBEGF</td>
<td>Heparin-binding epidermal growth factor</td>
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<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<td>Heparin/heparan sulfate-interacting protein</td>
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<td>Heparan sulfate proteoglycan</td>
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<td>IB</td>
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<td>Full Form</td>
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<td>IL</td>
<td>interleukin</td>
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<td>International unit</td>
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<td>IUGR</td>
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<td>IVF-ET</td>
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<td>PTX</td>
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<td>Peroxisome Proliferator-Activated Receptor</td>
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<td>Rous sarcoma virus</td>
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<td>Standard deviation</td>
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<td>Standard error</td>
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<td>SERPIN</td>
<td>Serine protease inhibitor</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>Taq</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<td>TEMED</td>
<td>N,N,N',N'-tetramethylethlenediamine</td>
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<td>TIMP</td>
<td>Tissue specific inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxyl methyl) aminomethane</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
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<tr>
<td>u-PA</td>
<td>urokinase-type plasminogen activator</td>
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PUBLICATION LIST

PEER REVIEW PAPERS


**ABSTRACTS AND ORAL PRESENTATIONS**

1. **Chou CS, MacCalman C D. and Leung PCK** (oral presentation) 2001 A novel Gonadotropin-Releasing Hormone Form (GnRH II) activate the MMPs/TIMP-1 cascade in Human Trophoblasts In Vitro. Canadian Fertility and Andrology Society; 2001 Oct 4-6: Whistler, BC.

2. **Chou CS, Tai CJ, MacCalman C D. and Leung PCK** (oral presentation) 2001 Both Gonadotropin-Releasing Hormone Forms (GnRH I and II) activate the MMPs/TIMP-1 cascade in Human Decidual Cells In Vitro. *Fertil and Steril* 76 : S-35.

3. **Chou CS, MacCalman C D. and Leung PCK** (poster presentation) 2002 GnRH II but not TGF-β1 or IL-1 β, up-regulates uPA mRNA expression in human decidual cells in vitro. *UBC/C&W Student Research Forum Poster Presentation, Children's & Women's Health Center of British Columbia*, Poster #56 (March 11, 2002)

4. **Chou CS, MacCalman C D. and Leung PCK** (oral presentation) The Role of GnRH I
And GnRH II in Regulating Urokinase Plasminogen Activators (uPA) And Inhibitors (PAI) system in Cultured Trophoblasts. *UBC/Dept of OB & GYN Research day presentation*, Chan Auditorium, BC Women's Hospital (April 12, 2002).


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1. Division of Reproductive Endocrinology and Infertility Research Award of UBC Department of Obstetrics and Gynaecology Research Day, 19 April 2001


3. Canadian Women’s Health Foundation Award For the best paper on Women’s Health of UBC Department of Obstetrics and Gynaecology 12.April, 2002


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PART I: 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 in favor of career establishment (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, their 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 fertilisation 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.

Recently, the administration of Gonadotropin releasing hormone (GnRH) agonists and antagonists during early pregnancy has been shown to improve pregnancy rates in women undergoing IVF-ET (Casper, 1991, Wilshire et al., 1993; Weissman A and Shoham Z, 1993) and in women diagnosed with unexplained, primary infertility (Smitz et al., 1991; Balasch et al., 1993). Although the use of GnRH analogs in these clinical settings is now widely accepted, the cellular mechanism(s) by which these synthetic compounds enhance pregnancy outcome is not fully understood. To address these outstanding issues, we have examined the ability of GnRH, and the second mammalian form of this hormone, GnRH II, and their antagonists to regulate the expression
of matrix metalloproteinases and urokinase plasminogen activator, two major classes of proteinases operative at the maternal-fetal interface, in primary cultures of human trophoblasts and decidual stromal cells isolated from first trimester tissues.

1.2 Implantation and Placentation in the Human

The establishment of a successful pregnancy is dependent on the coordinated development of the implanting embryo and the maternal endometrium (Tabibzadeh and Babaknia, 1995; Paria et al., 2000; 2002). In particular, the endometrium must have undergone a series of hormonally-regulated morphogenetic events in preparation for pregnancy. The blastocyst, in turn, must have attained the ability to interact with the diverse cell types that constitute the endometrium and subsequently form a functional placenta. As the endometrium is only receptive to the implanting embryo during a defined period of the menstrual cycle, known as the “window of implantation” (Hertig and Rock, 1956; Nikas, 1999; Wilcox et al., 1999), dysynchrony in the spatial or molecular development of the maternal and/or the fetal compartment often results in spontaneous abortion and, in less severe cases, defects in the formation and organization of the placenta that often compromise continuation of pregnancy to term (Paria et al., 2000, 2002; Kao et al., 2002).

1.2.1 Cyclic Remodelling of the Human Endometrium

The epithelial and stromal cells of the human endometrium undergo cyclic proliferation, differentiation and shedding in response to the gonadal steroids, 17\(\beta\)-estradiol (E2) and progesterone (P4) (Noyes et al., 1950). After menstruation, the endometrium regenerates under the influence of E2 to produce a dense cellular stroma containing narrow tubular glands and
small blood vessels. Immediately after ovulation, the effects of P4 on 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; if an embryo is 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 phenotype, followed by menstrual shedding.

The putative "window of implantation" in the human is believed to span cycle days 20-24 and involves the luminal epithelium and subsequently endometrial stroma (Hertig et al., 1956; Nikas, 1999; Wilcox et al., 1999). This receptive period is associated with distinct morphological and molecular changes in the luminal epithelium of the endometrium. 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 avb3 and a4b1) and the trophin-bystin/tastin complex have also been found to be temporally regulated in this endometrial cell layer within and framing the "window of implantation" (Aplin et al., 1995; Suzuki et al., 1999; Lessey et al., 2000, Kao et al., 2002)( Fig. 1). The mid secretory stroma also shows histological changes that represent the earliest cascade of differentiative events leading to decidualisation (Noyes et al., 1950). 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 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 persist. At this time, vascular differentiation occurs to produce prominent spiral arterioles surrounded by a cuff of pseudo-decidual cells, which are enlarged stromal cells that resemble the decidual cells of pregnancy.

Decidualization of the endometrium involves the differentiation of the stromal cells that acquire distinct morphological and functional features (Noyes et al., 1950). Morphological decidualisation is expressed histologically by a change to a polyhedral cell shape with an increase in cell size, and ultrastructurally by an extensive development of the organelles involved in protein synthesis and secretion, and by the appearance of desmosomes and gap junctions. Functionally, decidualization is associated with the onset of prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) secretion (Lala et al., 1984). A significant population of bone-marrow derived cells amounting to over 40% of the total and comprising large granular lymphocytes (LGLs), macrophages and to a lesser extent T cells, are now also present in the decidualised stromal compartment of the human endometrium (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). Mast cells have also been detected in the human decidua (Marx et al., 1999).
The diverse populations of cells that constitute the decidua allows this dynamic tissue to fulfill paracrine, nutritional, and immunoregulatory functions throughout pregnancy (Lala et al., 2002). In addition, the decidua plays a key embryo-regulatory role by virtue of its intrinsic ability to regulate the invasion of trophoblastic cells into the underlying maternal tissues and vasculature during early pregnancy. 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).
Growth Factors and cytokines

Regulation of changes in surface epithelium

Inner cell mass

EGF signaling

LIF signaling

Integrin, Mucin, Fibronectin, L-Selectin and Integrin

Regulation of prostaglandin production

Pinopodes

HB-EGF

BMP2

COX-2

PGI2, PGE2

Hoxa-10

Decidulization

Growth Factors and cytokines

Hormone production (Estrogens, Progesterone)

capillaries
Figure 1. Schematic representation of the molecular mechanisms of human embryo implantation. The diagram shows a peri-implantation-stage blastocyst (approximately six to seven days after conception) and the processes for uterine receptivity and blastocyst apposition and adhesion. Trophectodermal cells of the blastocyst are able to secrete several growth factors such as LIF, EGF and IL family to modify endometrial surface, which is thought to be necessary during the early process of embryo implantation. Simultaneously, endometrial epithelial cells might undergo some morphological changes, such as pinopodes. These are characterized by an apical microvilli retraction and the emergence of large apical protrusions of endometrial epithelium and express unique cell adhesion molecule(s) such as integrin, L-selectin and fibronectin that mediate an initial attachment between these cells.
Figure 2. Schematic representation of the molecular mechanisms of human blastocyst invasion. The diagram shows an invading blastocyst and the processes necessary for trophoblast invasion. Placentation in the uterine wall is the requisite step after implantation. The temporal and spatial expression of several growth factors and cytokines within the uterus regulates trophoblast proliferation and differentiation and subsequently controls cell migration and trophoblast invasion.
1.2.2 Placentation

The human placenta plays a key role in regulating the growth, development, and survival of the fetus during pregnancy (Boyd and Hamilton, 1970; Aplin, 1991). Errors in the formation of this transient organ are associated with fetal demise within the first two months of gestation, as is observed in pregnancies terminating in spontaneous abortion (Salafia et al., 1993; van Lijnschoten et al., 1994). Similarly, aberrant placental structure and function are observed in cases of intrauterine growth retardation and preeclampsia, both of which have deleterious effects on the maturation of the fetus (Khong et al., 1986; Krebs et al., 1996). The physiological role of the placenta may extend well beyond the gestational period, and recent studies have correlated the size of this organ at birth with the onset and incidence of specific diseases in adults (Barker et al., 1990; Barker, 1995).

The outer trophectodermal cell layer that gives rise to the epithelial cells of the human placenta is the first cell lineage that can be distinguished in the pre-implantation blastocyst (Hertig et al., 1956; Boyd and Hamilton, 1970). During implantation, these trophectodermal cells undergo apposition, attachment, and penetration through the surface epithelial cells and basement membrane of the maternal endometrium, allowing for the burrowing of the blastocyst into the uterine wall (Schlafke and Enders, 1975; Bentin-Ley et al., 2000) (Fig. 2). These early stages of development are critical to the establishment of a successful pregnancy. It is estimated that between 30% and 70% of human embryos are lost at this time of implantation (Cooke, 1988; Wilcox et al., 1988).

During the earliest post-implantation phases observed in the human, the embryonic trophectoderm consists of two discrete trophoblastic cell populations: mononucleate cytotrophoblasts and a multinucleated syncytial trophoblast (Hertig et al., 1956). The syncytial trophoblast is a terminally differentiated cell formed by the post-mitotic fusion of the underlying
villous cytotrophoblasts (Richart, 1961; Kliman et al., 1986). Although the trophoblastic cell subpopulation that is involved in the initial invasion of the maternal tissue remains unclear (Pijnenborg, 1990; Aplin, 2000), histological evidence suggests that both the syncytial trophoblast and cytotrophoblasts interact with the cells that constitute the endometrium (Enders, 1976). These trophoblastic cells are capable of remodelling the uterine environment and, following infiltration by a vascularized fetal mesenchyme, organize into mature chorionic villous structures. Chorionic villi are ultimately comprised of a mesenchymal core containing fetal blood vessels, a single layer of villous cytotrophoblasts that rests on a basement membrane, and an outer syncytial trophoblast layer that is in direct contact with the maternal endometrium and blood. The formation of chorionic villi has been described as the hallmark of human haemochorial placenta, as the fetal circulatory system is separated from the maternal blood cells by at least one layer of trophoblastic cells throughout all stages of pregnancy (Boyd and Hamilton, 1970).

Cytotrophoblasts at regions adjacent to the maternal tissue enter either of two distinct pathways of cellular differentiation: 1) the villous pathway in which these cells undergo terminal differentiation and fusion to form syncytium as described above or 2) the extravillous pathway (Morrish et al., 1998). Human extravillous cytotrophoblasts undergo extensive proliferation and breach the syncytial trophoblast layer, forming large cellular columns that extend into the maternal decidua and attach the placenta to the uterine wall during pregnancy (Enders, 1968; Muhlhauser et al., 1993). Subpopulation(s) of these extravillous cytotrophoblasts subsequently dissociate from the tips of this cellular column and invade deeply into the underlying maternal tissue (Pijnenborg et al., 1980). These extravillous cytotrophoblasts invade the uterine stroma and superficial myometrium as individual mononucleate cells and penetrate the basal lamina of the uterine vasculature. This results in the remodelling of the endothelial and smooth muscle cells in these blood vessels, thereby increasing blood flow to the placenta and
ensuring an adequate supply of nutrients and oxygen to the growing fetus (Brosens et al., 1967; Pijnenborg et al., 1983). In addition, large multinucleated trophoblastic cells, referred to as placental bed giant cells, are present in the decidua and myometrium during the first trimester of pregnancy and are most numerous at a time period that correlates with this phase of extravillous cytotrophoblast invasion (Pijnenborg et al., 1981; Al-Lamki et al., 1999).

At the end of the first trimester of pregnancy, the basic structure of the human placenta is well established and all the different trophoblastic cell subpopulations are present at the maternal-fetal interface (Boyd and Hamilton, 1970). Placental development beyond this time period involves continued trophoblastic cell proliferation and differentiation which leads to the subsequent growth and expansion of this organ until the end of the gestational period (Simpson et al., 1992). The placenta, along with the vast majority of trophoblastic cells, is normally expelled from the mother following the delivery of the fetus at term. Fetal blood vessels, a single layer of villous cytotrophoblasts that rests on a basement membrane, and an outer syncytiotrophoblast layer that is in direct contact with the maternal endometrium and blood.
Figure 3. Schematic representation of an implanted embryo and the processes necessary for the maintenance of an early pregnancy. The diagram shows placenta releases vascular endothelial growth factor (VEGF) to promote angiogenesis, and TGF, progesterone and human chorionic gonadotropin (hCG) to modulate immune response.
1.3: In vitro models for the Study of Human Implantation and Placentation.

Progress in our understanding of human implantation and placentation has been limited by the fact that in vivo human experimentation is not ethically feasible and the morphological differences between the human placenta and these of experimental and domestic animals (Leiser and Kaufmann, 1994). Consequently, most of our information regarding these two inter-related developmental processes has relied on histological studies of hysterectomy or term placental tissue specimens (Hertig et al., 1956; Boyd and Hamilton, 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 formation and organization of the human endometrium and placenta (Irwin et al., 1989; Morrish et al., 1998; Frank et al., 2000; King et al., 2000).

1.3.1: Trophoblastic Cell Model Systems

Human term placental tissues can be enzymatically dispersed and the trophoblastic cells purified by using either density gradients or immunoselection methods. These two methods result in the isolation of highly purified populations of cytotrophoblasts (Kliman et al., 1987; Yui et al., 1994; Morrish et al., 1997). Villous cytotrophoblasts isolated from human term placentae enter a program cellular differentiation that mimics many of the cellular events associated with chorionic villous formation in vivo. In particular, freshly isolated mononucleate cytotrophoblasts undergo aggregation, differentiation, and fusion to form large multinucleated syncytial structures with time in culture. The formation of multinucleated syncytium in these primary cell cultures is also associated with an increase in the secretion of the β subunit of hCG,
a peptide hormone that correlates with trophoblast differentiation and fusion in vivo (Hoshina et al., 1982).

Mechanically processed chorionic villous explants obtained from first trimester placental tissues give rise to a highly migratory trophoblastic cell population with time in culture (Yagel et al., 1988a; Graham et al., 1992; Irving et al., 1995). These trophoblastic cells proliferate and can be further propagated in culture. The cellular characteristics of these primary cell cultures and their ability to invade amniotic membrane explants or various components of the extracellular matrix (ECM) has led to the suggestion that this method isolates the subpopulation(s) of extravillous cytotrophoblasts that are capable of detaching from the first trimester cytotrophoblast cell columns and invading the underlying maternal tissue (Yagel et al., 1988b; Graham and Lala, 1991; Irving et al., 1995). This primary cell culture system has been used extensively to examine the molecular mechanisms that regulate extravillous cytotrophoblast differentiation and invasion in vitro (Lala and Hamilton, 1996).

Several choriocarcinoma cell lines that share many of the cellular characteristics of primary cultures of villous cytotrophoblasts and exhibit varying degrees of differentiation have also been established, two of the best characterized being BeWo cells which undergo and differentiation and fusion in response to cAMP and JEG-3 cells which are non-fusionogenic under the same culture conditions (King et al., 2000). Consequently, JEG-3 cells have been used as an in vitro model system to examine mononucleate trophoblastic cell biology.

1.3.2 Endometrial Cell Model Systems

The ability of gonadal steroids to regulate the cyclic remodeling processes that occur in the endometrium was first demonstrated using ovariectomised rodent model systems (Psychoyos, 1976). However, in contrast to the rat and mouse, embryonic implantation in the
human occurs at a time when the stream is not yet decidualised (Noyes et al., 1950; Hertig, 1956). 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).

The resident stromal and glandular epithelial cells of the human uterus 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 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 characterise 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 Implantation and Placentation

The highly regulated series of developmental processes that occur in the endometrium in preparation for pregnancy is believed to be mediated, at least in part, by alterations in the composition of the endometrial extracellular matrix (ECM) during the menstrual cycle, which is
the breakdown of these matrices during implantation and menstruation and the spatiotemporal
expression of cell surface receptors that modulate cell-cell or cell matrix interactions
(Tabibzadeh and Babknia, 1995; MacCalman et al., 1998). Similarly, the onset of trophoblast
differentiation along the villous or extravillous pathway is influenced by the ECM encountered
by these cells during the first trimester of pregnancy (Burrows et al., 1996; Xu et al., 2001;
Tarrade et al., 2002) (Fig. 1).

1.4.1: Extracellular matrix deposition

Regeneration of the endometrium during the proliferative phase of the menstrual cycle
involves the deposition of a scaffolding of ECM (Aplin et al., 1988; Mylona et al., 1995; Church
et al., 1996). The undifferentiated stroma produces ECM of classically mesenchymal
composition; 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. However, changes in the ECM accompany the
transition from undifferentiated stroma to decidua (Wewer et al., 1985; Kislaus et al., 1987;
Ruck et al., 1994). The decidual ECM lacks the bundles of uniform-diameter parallel fibrils
found in the intercellular spaces of the endometrial stroma. Fibril diameters and orientations are
variable and fibrils are sparsely distributed, though the major collagen types I, III, and V and
fibronectin are still present. Type VI collagen is now absent. The decidual cells encapsulate
themselves in a pericellular basal lamina through which pedicels protrude. The pedicels contain
secretory granules probably involved in the secretion of basement membrane components (Kislaus et al., 1988). 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). Thus, the differentiation of endometrial stromal ECM presents two contrasting molecular paradigms. The first is the selective removal during decidualization 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 decidualization 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 also 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 (Aplin et al., 1988; Ruck et al., 1994).
In the placenta, villous cytotrophoblasts form a polarized epithelial monolayer that is anchored to the underlying chorionic villous basement membrane. In the human, this basement membrane separates the trophoblastic cells from the villous mesenchyme and is comprised of several ECM components, including heparin sulfate proteoglycans, collagen type IV, and Ln (Earl et al., 1990; Damsky et al., 1992; Onodera et al., 1997). Several conflicting reports exist regarding the expression of Fn in the villous basement membrane, which may be partially explained by the different epitope-specific antibodies used to immunolocalize this glycoprotein or the different stages of gestation examined in these studies (Yamada et al., 1987; Virtanen et al., 1988; Earl et al., 1990; Damsky et al., 1992). However, Fn is expressed at high levels in the villous mesenchymal core at all stages of gestation examined to date. The composition of the villous mesenchymal ECM is not homogeneous throughout the chorionic villi of the human placenta. For example, tenascin, an ECM glycoprotein that exhibits anti-adhesive properties in vitro (Aufderheide and Ekblom, 1988), is expressed at sites of cytotrophoblastic cell proliferation and in regions below degenerating syncytium (Castelluci et al., 1991; Damsky et al., 1992). This has led to the proposal that tenascin modulates villous cytotrophoblast differentiation. It remains unclear whether tenascin plays a direct role in promoting trophoblast differentiation along the villous or extravillous pathway or is a by-product of the cellular proliferation and differentiation associated with these developmental processes.

The extravillous cytotrophoblast column appears to produce an ECM that is, at least in part, of trophoblastic origin and is comprised of ECM components that are typically associated with the basement membrane (Damsky et al., 1992; Huppertz et al., 1996; 1998; Rhode et al., 1998). In addition, oncofetal FN, which is a product of Fn mRNA variant, with a unique glycopeptide epitope (Matsuura and Hakomori, 1985; Matsuura et al., 1989), has been detected at sites of direct contact between this cellular column and the maternal decidua (Feinberg et al.,
This Fn isoform is synthesized and secreted by trophoblastic cells in culture (Feinberg et al., 1991) and modulates cytotrophoblastic column formation in vitro (Aplin et al., 1999). Collectively, these observations have led to the proposal that oncofetal Fn maintains placental-uterine interactions during pregnancy. The invasive extravillous cytotrophoblasts that have detached from the cellular column must then interact with the ECM and basement membrane of the decidua (Wewer et al., 1985; Aplin et al., 1988; Aplin et al., 1995).

The ECM has been shown to modulate the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta (Kao et al., 1988). For example, cytotrophoblasts cultured in the absence of serum fail to undergo aggregation and fusion with time in culture. However, the addition of Fn or Ln to these primary cell cultures has been shown to promote syncytial trophoblast formation in vitro. Fn and Ln appear to have different effects in trophoblastic cells isolated from the human first trimester placenta (Burrows et al., 1993). In these cultures, Ln promoted a non-motile phenotype whereas Fn resulted in an increase in cellular motility and the formation of multinucleated cells. These cells are capable of secreting both of these ECM components (Xu et al., 2000), suggesting that human trophoblastic cells possess intrinsic mechanisms to regulate cellular differentiation along the villous and extravillous pathway in vitro.
Figure 4. Schematic representation of the association between MMP family members and plasmin.

MMPs can be activated by cleavage of the propeptide by plasmin or by members of the MMP family. MMPs degrade most components of the ECM. Plasminogen activators -tissue type (t-PA) and urokinase-type (u-PA)-are able to convert plasminogen to plasmin. Plasmin is an extremely potent protease that can by itself degrade virtually all extracellular matrix proteins. Both plasmin and MMPs appear to participate in trophoblast invasion.
1.4.2: Extracellular matrix degradation—proteinases and their inhibitors

A large proportion of endometrial remodelling seen in both the proliferative and secretory phases of the menstrual cycle involves the degradation of ECM components, particularly interstitial collagens and basement membranes (Yagel et al., 1988b; Strickland and Richards, 1992; Lala and Hamilton, 1996; Bischof et al., 2000). The decidua of early pregnancy is also subject to further degradation by the invading trophoblasts that have been shown to adopt similar cellular mechanisms for ECM degradation to those observed during tumour cell invasion. Consequently, proteinases and their associated inhibitors are believed to play a key role in human implantation. Two major classes of proteinases have been studied in human trophoblast and endometrial cells: plasminogen activators and matrix metalloproteinases.

1.4.2-A: Plasminogen activators and their inhibitors

The plasminogen activators are substrate-specific serine proteinases that mediate cleavage of plasminogen to plasmin, which exhibits a broad range of serine protease activity (Vasselli et al., 1991; Andreasen et al., 2000) (Fig. 4). The proteinase activator system includes the urokinase-type plasminogen activator (uPA), the tissue-type PA (tPA), the PA inhibitor-1 and -2 (PAI-1 and PAI-2, respectively) and the uPA receptor.

uPA has been shown to be produced by human trophoblastic cells in vitro and in vivo (Astedt et al., 1986; Queenan et al., 1987; Yagel et al., 1988b; Hofmann et al., 1994). In addition, the membrane-bound uPA receptor is expressed by the invasive extravillous cytotrophoblasts during the first trimester of pregnancy (Multhaupt et al., 1994; Pierleoni et al., 1998). The inhibition of uPA activity in extravillous cytotrophoblasts, using neutralizing antibodies specific for this proteinase or by increasing the endogenous production of PAI-1, inhibits the invasive capacity of these primary cell cultures (Yagel et al., 1988b; Graham et al., 1998).
1994; 1997). Taken together, these observations suggest that uPA plays a key role in regulating extravillous cytotrophoblast invasion during human implantation.

The expression of PAI-1 and PAI-2 appears to be differentially regulated during the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta (Feinberg et al., 1989). In particular, PAI-1 expression is high in freshly isolated mononucleate cytotrophoblasts whereas maximal PAI-2 expression is detected in the multinucleated syncytial structures that form in these cultures at later time points. The functional significance of this switch in PAI subtype expression in these primary cell cultures remains to be determined.

1.4.2-B: 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 remodelling processes (Woessner, 1991; Fata et al., 2000). To date, 26 distinct members of the MMP gene family been identified (Fig. 5). 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)(Fig. 4). 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 proteolytic 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 also allow it to bind to heparan-sulphate-containing proteoglycans and possibly chondroitin-sulphate-containing proteoglycans in the ECM (Yuet 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., 2000; 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 the expression of MMP-2 and
MMP-9 occurs in this dynamic tissue at term (Xu et al., 2002). To date, the cellular localization of only some of these MMP subtypes in the human endometrium has been determined. During the follicular phase, MMP-1, MMP-2 and MMP-3 have been detected in the stroma, MMP-7 and MMP-9 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, while MMP-7 is in the glandular epithelium, and MMP-9 is in the glandular epithelium and neutrophils (Rodgers et al., 1994; Irwin et al., 1996; Jeziorska et al., 1996). Within menstrual tissue, MMP-1 and MMP-3 have been detected in stromal cells near blood vessels, MMP-2, MMP-9, MMP-10 and MMP-11 are in the stroma, MMP-7 is in the glandular epithelium and MMP-9 is 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 has not been examined in this dynamic tissue (Fata eta al., 2000; Curry et al., 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, localized increases in TIMP-1 mRNA and protein expression have 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 Salmonsen, 1999). Similarly, TIMP-2 mRNA and protein expression levels were found to be higher in the vasculature than in glandular epithelium, stroma or deciduas of early pregnancy (Hampton and Salmonsen 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 decidualization and/or play a critical role in regulating trophoblast invasion (Zhang and Salmonsen, 1999; Goffin et al., 2003). Therefore, it appears that MMP expression levels are involving large regulated fluctuations in the epithelial and/or stromal cells of the endometrium and in small foci within these cellular compartments whereas TIMP levels remain relatively constant during the menstrual cycle.

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 endometrium 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 (Salmonsen et al., 1997). The addition of P4, but not E2, to the culture medium of these primary cell cultures is capable of causing a significant decrease in the levels of these MMP subtypes and concomitant increase in TIMP expression levels (Martelli et al., 1993; Osteen et al., 1994; Higuchi et al., 1995; Marbaix et al., 1995, 1996; Schatz et al., 1999). In contrast, the withdrawal of gonadal steroids from the culture medium of endometrial stromal cells allowed them to undergo steroid-mediated decidualisation, and this culture model system is believed to recapitulate the cellular mechanisms underlying menstruation, resulting 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 (Salmonsen et al., 1997).

Human trophoblastic cells also produce multiple MMP subtypes, including MMP-1, -2, -3, -7, -9, -11, and -14 (Fisher et al., 1989; Moll and Lane, 1990; Librach et al., 1991; Auto-Harmainen et al., 1992; Polette et al., 1994; Nawrocki et al., 1996; Vettraino et al., 1996; Huppertz et al., 1998; Hurskainen et al., 1998; Sawicki et al., 2000). MMP-2 is expressed by the invasive extravillous cytotrophoblasts in vivo (Polette et al., 1994) and is produced by
extravillous cytotrophoblasts isolated from the human first trimester placenta (Graham et al., 1993; Xu et al, 2000). MMP-2 activity can also be regulated by a multimeric protein complex containing MMP-14, also known as membrane type-MMP-1 (MT-MMP-1)(Sato et al., 1994; Young et al., 1995). Interestingly, MT-MMP-1 is also expressed by human extravillous cytotrophoblasts in vivo, suggesting a potential mechanism for regulating MMP-2 activity during trophoblast invasion (Narwocki et al., 1996; Hurskainen et al., 1998).

MMP-9 is expressed in human extravillous cytotrophoblasts in vivo and has been shown to be up-regulated during cytotrophoblast invasion in vitro (Fisher et al., 1989). Function-perturbing antibodies specific for MMP-9 are capable of reducing the invasive capacity of these trophoblastic cells in vitro (Librach et al., 1991). In addition, the production of MMP-9 is down-regulated during the third trimester of pregnancy, paralleling the decline in trophoblast invasiveness associated with gestational age (Polette et al., 1994; Shimonovitz et al., 1994). Collectively, these observations have led to the proposal that MMP-9 is a key regulator of extravillous cytotrophoblast invasion during human implantation. This hypothesis is supported by recent observations demonstrating a reduction in MMP-9 activity in cytotrophoblasts isolated from placentae diagnosed with preeclampsia (Graham and McCrae, 1996), a disease in which trophoblast invasion into the maternal tissue and vasculature is believed to be compromised (Khong et al., 1986; Robertson et al., 1986; Redline and Patterson, 1995).

MMP-2 and -9 are also believed to play a role in chorionic villous formation. These two proteinases are produced by the syncytial trophoblast in vitro and in vivo and are secreted basolaterally towards the fetal mesenchymal core (Sawicki et al., 2000). This polarized release is believed to contribute the rapid expansion of the mesenchymal core during later stages of gestation. Previous studies have also demonstrated the expression of MMP-1, -3, and -7 in the
trophoblastic cells of human chorionic villi (Vetraino et al., 1996). However, the functional role of these MMPs during this developmental process remains to be elucidated.

Human trophoblastic cells also produce TIMP-1, -2, and -3 suggesting both an autocrine and paracrine regulation of MMP activity at the maternal-fetal interface (Graham and Lala, 1991; Polette et al., 1994; Higuchi et al., 1995; Hurskainen et al., 1996; Ruck et al., 1996). The ability of TIMP-1 and -2 to inhibit trophoblast invasion has been demonstrated in vitro (Graham and Lala, 1991; Librach et al., 1991). Although TIMP-2 can serve as an inhibitor of MMP-2 activity, the recruitment of this secreted protein into a complex with MT-MMP-1 can also activate this proteinase (Strongin et al., 1993; 1995; Young et al., 1995). Previous studies have demonstrated that TIMP-3 expression is up-regulated during trophoblast invasion in vitro (Bass et al., 1997). The expression levels of TIMP-3 are co-ordinate regulated with MMP-9 expression in these primary cell cultures, suggesting that an intricate balance between the production of proteases and their inhibitors modulates the over-all proteolytic activity of trophoblastic cells during human implantation.
A. MMPs with a minimal domain (MMP-7)

B. MMPs with a Hemopexin/Vitronectin domain (MMP-1, MMP-3, MMP-8, MMP-10, MMP-12, MMP-13, MMP-18, MMP-19, MMP-20)

C. MMPs containing a Furin-cleavage site (MMP-11)

D. Transmembrane MMP with a Furin-cleavage site (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25)

E. MMP containing a Fibronectin type II repeat (MMP-2, MMP-15, MMP-9)

- Pre-peptide
- Catalytic domain
- Hemopexin/Vitronectin domain
- Pro-domain
- Zinc binding site
- Cysteine switch
- Hinge region
- Transmembrane and cytoplasmic domains
- Furin-cleavage site
- Fibronectin type II repeat
Figure 5. Structural domains of the matrix metalloproteinases. Diagrams of the domain structure of the different subgroups of the matrix metalloproteinase family. The prodomain contains a highly conserved sequence with an unpaired cysteine sulfhydryl group whose interaction with the active site zinc maintains the enzyme in latent form (the cysteine switch). (A) MMPs with a minimal domain. (B) MMPs with a hemopexin/vitronectin domain. The prodomain of some MMPs contains a recognition motif for furin-like enzymes (C), whose cleavage results in activation of the MMP. MMPs containing a hemopexin/vitronectin domain and transmembrane MMP with a Furin-cleavage site (D). The catalytic domain of the gelatinases (MMP-2 and MMP-9) contains three gelatin-binding fibronectin type II repeats (E). The hemopexin/vitronectin domain is folded into a four-bladed propeller structure.
1.5 Regulation of Human Implantation and Placentation

Although it has been well established that E2 and P4 are key regulators of the dramatic morphological changes that occur in the human endometrium during each menstrual cycle, there is increasing evidence to suggest that other factors are involved in creating an environment that promotes and/or supports a viable pregnancy. For example, prostaglandins (PGs) are believed to be involved in the initiation and maintenance of stromal cell decidualisation in the rodent and human endometrium in vitro and in vivo (Kennedy et al., 1982; Frank et al., 2000). In particular, prostaglandin E2 (PGE2) has been shown to potentiate the stimulatory effects of E2 and P4 on the PRL secretion of cultured human endometrial stromal cells (Challis et al., 2002). As the glandular epithelium of the human endometrium secretes high levels of PGE2 during the secretory phase of the menstrual cycle, it has been suggested that this hormone plays a key role in vascular permeability (Psychoyos et al. 1995; Kao et al., 2002).

Several growth factors and their receptors including epidermal growth factor (EGF), insulin-like growth factor-II (IGFII), and transforming growth factor-β1(TGF- β 1) have been detected in the human endometrium and placenta (Giudice, 1994). The spatiotemporal expression of these growth factors suggest that they play a central role in human implantation by regulating the decidualization of the endometrial stroma and/or the differentiation of trophoblasts along the invasive or non-invasive pathways in an autocrine and/or paracrine manner. For example, IGF-II has been detected in the endometrial stroma during the late secretory phase, and in the decidua and extravillous cytotrophoblast columns of the first trimester placenta in vivo (Han et al., 1996) and has been shown to stimulate trophoblast invasion in vitro (Irving and Lala, 1995). The stimulatory effects of IGF-II on trophoblast invasion were inhibited by IGF-binding protein (IGFBP)-1, a major product of the decidua of early pregnancy (Han et al., 1996). Similarly, transforming growth factor- β 1 (TGF- β 1), which is produced by the placenta and decidua in vivo (Graham et al., 1992; Lysiak et al., 1995), is
capable of reducing proliferation and promoting the aggregation, differentiation, and fusion of these isolated extravillous cytотrophoblasts in vitro (Graham et al., 1992; 1994). Finally, EGF, which is produced by both the trophoblastic cells and decidual cells at the maternal-fetal interface (Hofmann et al., 1992, 1994; Leach et al., 1999), is capable of potentiating the formation of multinucleated syncytium in these primary cells cultures (Morrish et al., 1997; 1998). Furthermore, a decrease in EGF receptor activity has been shown to reduce the number of multinucleated cells that formed in these primary cultures (Rebut-Bonneton et al., 1993) (Fig. 1).

Collectively, these observations not only suggests that multiple factors act in concert to coordinate the morphological and molecular development of the decidua and/or the differentiation of trophoblasts along the invasive or non-invasive pathway, but also that a regulatory hierarchy capable of controlling the progression of these two inter-related must be operative at the maternal-fetal interface. The identification of one or more key regulatory elements will provide an opportunity to design diagnostic tests for patients with infertility and endometrial disorders. They may also target drug discovery for treating implantation-based infertility, as well as other endometrial disorders involving altered cellular proliferation and/or differentiation such as cancer, and endometrial-based contraception. Activin A has been shown to be capable of regulating the proliferation, differentiation and invasive capacity of benign and malignant cells of the prostate, breast and endometrium in vivo and in vitro, either directly or indirectly through the modulation of growth factor-mediated effects (Motta et al., 1996; Grundker et al., 2002; Chen et al., 2002; Sakamoto et al., 2003).

1.6 GnRH I, GnRH II and GnRHR Expression in Human

GnRH is present in all vertebrates and some non-vertebrate brains, where it acts to regulate reproduction in both males and females (Amoss et al. 1971). To date, 16 novel forms of GnRH have been identified in all species that span the evolutionary scale suggesting that the structure and function of these hormones are highly conserved. The biological actions of these
hormones have been well conserved for at least 500 million years of vertebrate evolution (Kasten et al. 1996). To date, there are at least 13 forms of GnRH identified in vertebrates (Carolsfield et al. 2000), and they are distributed in a range of tissues (Sherwood et al. 1993; King et al., 1995, Kasten et al. 1996). All forms of GnRH are comprised of ten amino acids, with conserved amino acid residues at positions 1, 2, 4, 9, and 10, but are highly variable at residues 5 to 8.

In humans, like all other mammals, the classical mammalian form of hypothalamic GnRH (GnRH I) is a key regulator of reproduction (King et al., 1995; Sealafon et al., 1997). It is widely accepted that after its release from the mediobasal hypothalamus, this decapeptide is transported via the portal circulation to the anterior pituitary where it stimulates the synthesis and secretion of Follicle Stimulating Hormone (FSH) and Luteinizing hormone (LH), two hormones that play pivotal roles in the regulation of reproductive performance in both males and females. However, the identification of low affinity/high capacity binding sites in extrapituitary tissues including placenta, endometrium, breast, testis and ovary suggest that in addition to this classical pathway, GnRH may have direct regulatory actions on the development and function of these reproductive tissues (Khodr and Siler-Khodr, 1980; Casan et al., 1998; Raga et al., 1998; Chen et al., 2002).

Recent studies have demonstrated that a distinct gene exists encoding a second form of GnRH, which has been termed GnRH II to distinguish it from the classical mammalian form (GnRH I), is expressed in the human and other primates (Leschied et al., 1997; White et al., 1998). The GnRH-II gene is located on chromosome 20, corresponding to band 20p13(White 1998), while GnRH-I is located at 8p21-p11.2(Yang-Feng et al 1986). The human GnRH-II gene is much shorter (2.1 kb) than the GnRH-I (5.1 kb) gene (Hayflick et al, 1989). However, both genes share structural similarities. Both the new human GnRH-II gene and the GnRH-I gene have four exons and the predicted preprohormone encoded by human GnRH-II is organized identically to that of other GnRHs: all have a signal sequence, followed by a GnRH decapeptide, a conserved proteolytic site (Gly-Lys-Arg), and a GnRH associated protein(GAP).
The corresponding preprohormone elements of the two human GnRHs are quite similar in length, with the striking exception that the GAP is 50% longer in GnRH-II than in GnRH-I (84 vs. 56 amino acids). A similar disparity in the GAP was recently reported for tree shrew GnRH I and GnRH II (76 vs. 56 amino acids), suggesting that a relatively larger GAP may be common among mammalian GnRH-II forms. (Fig. 6)

In contrast to GnRH-I, GnRH-II, which was first identified in the chicken (cGnRH-II), is expressed primarily in extra-pituitary tissues in the human (Leschied et al., 1997; White et al., 1998). As GnRH-II has been conserved throughout vertebrate evolution, it has been proposed that this form of GnRH is biologically significant (Leschied et al., 1997; Temple et al., 2003). To date, the biological function(s) of GnRH-II in the human remain poorly characterised. However, there is increasing evidence to suggest that GnRH-II has a distinct regulatory role in gonadotropin secretion (Neill, 2002) and may act as a potent tumor suppressor (Chen et al., 2002, Choi et al., 2002; Grundker et al., 2002).

The detection of two distinct forms of GnRH in human tissues suggests that multiple forms of the GnRH receptor (GnRHR) may also be expressed. Human GnRHR is encoded by a single gene located on chromosome 4q (Fan et al., 1995). The coding region is divided by 3 exons and 2 introns splice the open reading frame. Exon II contains 219 bp and the remainder of the approximately 5 kb transcript is distributed between exons I and III. The complete coding region represents only 987 bp leaving an extensive 5' and 3' non-translated region. The deduced 328 residue protein sequence includes with a seven transmembrane domain topology characteristic of G-protein-coupled receptors. At the 3' end of the GnRHR gene, five classical polyadenylation signals are found scattered throughout an 800bp region. Five putative promoters and transcription initiation sites have been identified in the 5' end of the GnRHR
gene suggesting that the transcriptional regulation of this gene may be complex and involve tissue specific regulatory factors.

To date, two genes encoding a putative GnRH-II receptor (GnRHR-II), both of which exhibit significant nucleotide sequence homology to GnRHR, have been identified in the human genome (Millar et al., 1999, 2001; Neill et al., 2002). However, one of these genes, located on chromosome 14, encodes an antisense mRNA transcript (Millar et al., 1999) whereas a stop codon has been identified in the nucleotide sequence of exon II of the other putative GnRHR-II gene located on chromosome 1 (Neill et al., 2001; Millar et al., 2001). If it were not for the presence of this stop codon, it has been predicted that this gene could generate a full-length 7TM receptor protein. Although it is possible that post-transcriptional modification of this gene product may generate a functional GnRHR-II, only incompletely processed GnRHR-II mRNA transcripts have been detected in human tissues and cells (Neill, 2002). The identification of low and high affinity binding sites for both GnRH-I and -II on the surface of normal and metaplastic ovarian and endometrial cells warrants further investigation into the structure of the functional human GnRHR-II (Chatzaki et al., 1996; Grundker et al., 2002).
Comparison between GnRH I and GnRH II

- **Peptide:**
  - \(\text{Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH}_2\) (GnRH I)
  - \(\text{Gln-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH}_2\) (GnRH II)

**Chromosome:**
- GnRH I 8p21-p11.2
- GnRH II 20p13

**Genomic DNA**
- GnRH I 5.1 kb
- GnRH II 2.1 kb

![Diagram of gene organization between GnRH I and GnRH II](image)

**Figure 6.** Schematic comparison between GnRH I and GnRH II in gene organization

GnRH-I is spatiotemporally expressed during the development of the human embryo in vitro (Raga et al., 1998; Casan et al., 1999). In particular, GnRH-I is expressed in all of the blastomeres of the early cleavage stage human embryo (morula) but is localized to the trophectoderm of the blastocyst. Similarly, GnRH-I has been detected in the culture medium of developing rhesus monkey embryos. As significantly lower levels of GnRH-I were secreted by embryos with impaired development (Raga et al., 1996), these observations suggest that this peptide hormone plays a key role in the early stages of embryogenesis and may serve as a useful marker of embryo quality/viability. In support of this hypothesis it has recently been demonstrated that the addition of GnRH-I to the culture medium enhances the development of bovine and murine embryos in vitro whereas GnRH antagonists inhibit the development of these embryos (Yang et al., 1995; Lin et al., 1995 Raga et al., 1999). The inhibitory actions of GnRH antagonists on embryonic development were reversed by GnRH agonists in a dose-dependent manner, suggesting that these effects were receptor-mediated (Raga et al., 1992).

In the human placenta, GnRH-I has been localised to the villous cytotrophoblasts and syncytial trophoblast with maximum levels being observed during the first trimester of pregnancy (Lin et al., 1995). The ability of GnRH-I to regulate hCG production by placental tissues in an autocrine and paracrine manner has been demonstrated in vitro and in vivo (Siler-Khodr et al., 1986; Petraglia et al., 1987). These effects appear to be receptor-mediated since GnRH-antagonists are capable of inhibiting the stimulatory actions of GnRH-I and GnRH agonists in a dose-dependent manner. In addition, GnRH-I and/or GnRH-agonists have been shown to increase the secretion of prostaglandin E2 and F2α, P4, E2 and estriol by chorionic villous explants in vitro (Petraglia et al., 1987; Siler-Khodr et al., 1997). As these hormones play key roles in the decidualization of endometrial stromal cells, these observations provide further evidence to suggest that the placenta influences the developmental processes which occur in the human endometrium in preparation for the implanting embryo.
GnRH-I has been detected in the glandular epithelium and stroma of the human endometrium at all stages of the menstrual cycle (Casan et al., 1998; Raga et al., 1998). However, maximum levels of GnRH-I mRNA transcript and protein expression were detected in the glandular epithelium and stromal cells of the secretory endometrium (Dong et al., 1998). To date, the biological function(s) of endometrial GnRH-I has not been determined. The presence of low binding affinity/high capacity binding sites of GnRH-I in the normal endometrium and endometrial carcinomas suggests that GnRH-I may play a direct, regulatory role in the cyclic remodeling processes which occur in this dynamic tissue (Bourgain et al., 1994). In support of this hypothesis, immature mice immunised with antibodies directed against a peptide corresponding to amino acids 5-17 of the murine GnRHR resulted in the specific regression/inhibition of endometrial cell proliferation (Asirvatham et al., 1994). Recent studies have also demonstrated that GnRH-I is capable of decreasing TIMP-1 and TIMP-3 expression in human endometrial stromal cells undergoing steroid-mediated decidualisation in vitro (Raga et al., 1999). The ability of GnRH-I to regulate the synthesis and secretion of TIMP-1 and TIMP-3 in these primary cell cultures appears to be mediated by GnRHR as this inhibitory effect could be reversed by GnRH-antagonists in a dose-dependent manner. These studies suggest that endometrial GnRH-I plays a key role in human implantation and placentation by promoting the MMP-mediated invasion of extravillous cytotrophoblasts into the decidua.

Although it has been well documented that GnRH-I is capable of regulating hCG production in the embryo and first trimester placenta, the role(s) of this peptide in the formation and organization of the human placenta and/or the decidualisation of human endometrial stromal cells have not been defined.
1.6.2 Intracellular Signalling Pathways mediated by GnRHR (Fig. 7)

There is increasing evidence to suggest that the nature of the intracellular signalling initiated by GnRH binding to its receptor may vary from cell to cell. In pituitary-derived αT3 cells, GnRH-GnRHR interactions leads to the stimulation of two members of the family of GTP-binding proteins (G-proteins), Gq and/or G11 (Shah and Millligan, 1994) whereas in other cell types, the GnRHR may couple to other G proteins. Recent studies suggest that GnRHR in prostate cancer cells (LnCaP) is coupled to Gi and in SFG cells (insect SF9 cells expressing GnRHR), GnRH signals through Gs (Delahaye et al., 1997; Limonta et al., 1999) although additional components may be involved. Direct evidence for the activation of multiple G-proteins by GnRHR is still missing.

Downstream of G-proteins, several signaling processes have been reported to mediate GnRHR signaling. GnRHR-mediated activation of Gq in αT3 cells which then activates phospholipase C (Naor, 1990). This activation then leads to enhanced phosphoinositide turnover, production of diacylglycerol (DAG) and activation of various protein kinase C (PKC) subspecies. In addition, GnRHR has also been shown to activate 4 distinct mitogen-activated protein kinase (MAPK) signaling cascades; ERK, JNK, p38MAPK and BMK (Cheng and Leung 2000). However, the JNK signaling cascade appears to be the main MAPK pathway involved in GnRH signaling in alpha-T3-1 cells. GnRH stimulates JNK activity in these cells by a pathway that induces sequential activation of PKC, c-Src, Rac and MEKKI and/or elevated levels of calcium that are PKC-independent (Mulvaney et al., 2000). A possible explanation for these contradictory findings is that under distinct conditions, the relative role of the PKC-dependent pathway is altered. Activation of ERK by GnRHR in alpha-T3-1 cells also involves 2 distinct signaling pathways (Benard et al., 2001). The main pathway involves the direct activation of Raf I by PKC whereas the second, supportive pathway involves activation of Ras in a c-Src-and
dynamin-dependent manner. Activation of the MAPK cascades is also believed to play a role in the transcription of genes under the regulation of GnRH (Strahl et al., 1998).

Although ERK and JNK are activated in non-pituitary cells, the mechanism that induces this activation appears to vary and involve additional signalling components. For example, GnRH activates the MAPK cascade though a PKC-dependent pathway and seems to be coupled to the Gq protein in granulosa-luteal cells, as in pituitary cells (Kang et al., 2001) whereas ERK activation is mediated by both PKA and PKC (Han and Conn, 1999) in GGH3 cells. In Caov3 cells, GnRH signals either through Go or the Gβγ dimer and its effect on JNK activation is not dependent on PKC in LβT2 cells (Yokoi et al., 2000). Furthermore, in GnRHR-expressing COS-7 cells, GnRHR mainly transmits its signals to ERKs and JNK by transactivating the EGF receptor, which in turn activates several signaling pathways including c-Src, with direct activation of Ras or direct association and activation of PI3K (Stephens et al., 1997; Kraus et al., 2003).

The identification of distinct intracellular signaling pathways elicited by GnRHR may provide useful insight into the different biological effects observed for GnRH on pituitary cells and extra-pituitary tissues.
Figure 7. Schematic representation of proposed GnRHR signal transduction pathways. The GnRH-R mediates signaling events, including stimulation of calcium influx and activation of phospholipase C, leading to increased protein kinase activity. However, GnRH II-mediated signaling events still remain unclear.
1.6.3 Desensitization

Sustained stimulation of G-protein-coupled receptors (GPCRs) typically causes receptor desensitisation, which is mediated by phosphorylation, often within the C-terminal tail of the receptor.

The type I GnRH receptors (GnRH-Rs), found only in mammals, are unique in that they lack C-terminal tails and apparently do not undergo agonist-induced phosphorylation or bind beta-arrestin; they are therefore resistant to receptor desensitisation and internalise slowly. In contrast, the type II GnRH-Rs, found in numerous vertebrates, possess such tails and show rapid desensitisation and internalisation, with concomitant receptor phosphorylation (within the C-terminal tails) or binding of beta-arrestin, or both. Although type I GnRH-Rs do not desensitise, sustained activation of GnRH-Rs causes desensitisation of gonadotrophin secretion, and GnRH can cause down-regulation of inositol (1,4,5) trisphosphate receptors and desensitisation of Ca(2+) mobilisation in pituitary cells. The atypical resistance of the GnRH-R to desensitisation may underlie its atypical efficiency at provoking this downstream adaptive response. GnRH-Rs are also expressed in several extrapituitary sites, and these may mediate direct inhibition of proliferation of hormone-dependent cancer cells. Infection with type I GnRH-R-expressing adenovirus facilitated expression of high-affinity, PLC-coupled GnRH-R in mammary and prostate cancer cells, and these mediated pronounced antiproliferative effects of receptor agonists. No such effect was seen in cells transfected with a type II GnRH-R, implying that it is mediated most efficiently by a non-desensitising receptor. Thus it appears that the mammalian GnRH-Rs have undergone a period of rapidly accelerated molecular evolution that is of functional relevance to GnRH-Rs in pituitary and extrapituitary sites.

Phosphorylation is the most rapid means of GPCR desensitization; it is achieved rapidly after agonist stimulation by two classes of serine/threonine protein kinases including: 1) cAMP-
dependent protein kinase A and/or protein kinase C; and 2) the G-protein coupled receptor
kinases, GRKs (Premont et al. 1995).

1.7 Mitogen-activated protein kinase cascade

Protein kinases are enzymes that covalently attach phosphate to the side chain of either serine,
threonine, or tyrosine of specific proteins inside cells. Such phosphorylation of proteins can
control their enzymatic activity, their interaction with other proteins and molecules, their
location in the cell, and their propensity for degradation by proteases. MAPKs phosphorylate
specific serines and threonines of target protein substrates and regulate cellular activities ranging
from gene expression, mitosis, movement, metabolism, and programmed death.

MAPKs, a family of serine/threonine protein kinases that are rapidly activated in
response to a wide variety of stimuli. It is now clear that the MAPK cascade can be activated not
only by growth factor or cytokine receptors, but also by a variety of G protein-coupled receptors
(Wan et al., 1996, Dello Rocca et al., 1997). Stimuli for their activation include growth factors,
many of which have receptors with intrinsic protein tyrosine kinase activity. MAPKs are
involved in transmitting extracellular growth and differentiation signals into the cell nucleus,
resulting in an array of transcriptional and mitogenic effects. Recently, several groups have
demonstrated that GnRHR activation resulted in activation of the MAPK cascade, including
ERK (Sim et al., 1995, Sundaresan et al., 1996, Reiss et al., 1997), c-jun N-terminal protein
kinase (JNK) (Levi et al., 1998), and p38 MAP kinase (Roberson et al., 1999).
1.7.1 ERK1 and ERK2

ERK1 and ERK2 are widely expressed and are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors, transforming agents, and carcinogens, activate the ERK1 and ERK2 pathways. ERKs 1 and 2 are both components of a three-kinase phosphorylation module that includes the MKKK c-Raf1, B-Raf, or A-Raf, which can be activated by the proto-oncogene Ras (Gary et al., 2002). Mutations that convert Ras to an activated oncogene are common oncogenic mutations in many human tumors. Oncogenic Ras persistently activates the ERK1 and ERK2 pathways, which contributes to the increased proliferative rate of tumor cells.

1.7.2 JNK1, JNK2 and JNK3

The JNKs were isolated and characterized as stress-activated protein kinases on the basis of their activation in response to inhibition of protein synthesis (Kyriakis, et al., 1994). The JNKs were then discovered to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity. c-Jun is a component of the AP-1 transcription complex, which is an important regulator of gene expression. AP-1 contributes to the control of many cytokine genes and is activated in response to environmental stress, radiation, and growth factors. JNKs are important in controlling programmed cell death or apoptosis (Tournier, et al., 2000).

1.7.3 p38 Kinases

The importance of MAPKs in controlling cellular responses to the environment and in regulating gene expression, cell growth, and apoptosis has made them a priority for research related to many human diseases. It has been shown that p38 has roles in coupling hypoxic-
ischemic neuronal insults to activate p53 in neurons (Zhu et al., 2002). The requirement of early p53 activation for p38 MAPK activation and apoptosis in hepatoma cells indicates a functional regulation (link) between p53 and p38 MAPK (Kwon et al., 2002). Moreover, the stress-responsive p38 MAPK enhances p53 activity by phosphorylation and leads to cell cycle arrest or apoptosis (Takekawa et al., 2000).

1.8 GnRH regulates the activity of transcription factors AP-1 and p53

Both c-fos and c-jun transcripts are known to be regulated by GnRH in gonadotropes (Cheng et al., 2000, Wurmbach et al., 2001, Yuen et al., 2002). GnRH-agonist and antagonist have also been demonstrated to increase p53 levels in normal tissues or tumors (Gao et al., 2002; Tang et al., 2002).

1.8.1 transcription factors AP-1

AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. AP-1 is not a single protein, but a menagerie of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families, which recognize either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3') (Chimenov et al., 2001). Among these subgroups, c-Jun is the most potent transcriptional activator in its group (Ryseck et al., 1991). The Fos proteins, which cannot homodimerize, form stable heterodimers with Jun proteins and thereby enhance their DNA binding activity. AP-1 activity is induced by growth factors, cytokines, neurotransmitters, polypeptide hormones, cell–matrix interactions, bacterial and viral infections,
and a variety of physical and chemical stresses. These stimuli activate mitogen activated protein kinase (MAPK) cascades (Chang et al., 2001) that enhance AP-1 activity through the phosphorylation of distinct substrates.

JNK-mediated phosphorylation of c-Jun partially stimulates cell proliferation, as c-jun^{Ala63/73} fibroblasts have a proliferation defect that is less severe than that of c-jun^{-/-} fibroblasts (Behrens et al., 1999). c-Jun induction is also required for cell cycle re-entry of UV-irradiated fibroblasts (Shaulian et al., 2000). Importantly, c-Jun functions as a direct repressor of p53 gene transcription, and therefore p53 is elevated in c-jun^{-/-} fibroblasts through increased p53 gene transcription (Schreiber et al., 1999). Furthermore, c-Jun also decreases the transcriptional activity of p53 itself, down-modulating its ability to activate the p21^{Cip1} gene (Shaulian et al., 2000). Therefore, c-Jun can stimulate cell cycle progression through two mechanisms: induction of cyclin D1 transcription and repression of p21^{Cip1} transcription.

Recent evidence showed that in the absence of c-Jun, JunB may function as a positive growth regulator. However, in the presence of c-Jun, JunB has opposite effects, and this suggested that the differences in JunB and c-Jun function may be cell-type-specific or depend on the relative expression levels of these proteins. In support of this replacement of c-jun by junB prevents most of the developmental and cellular defects associated with a loss of c-Jun (Passegue et al., 2002).

AP-1 functions as a homeostatic regulator that keeps cells in a certain proliferative steady state. Changes in environmental conditions may enhance AP-1 activity. Robust or persistent activation of AP-1 in cells containing damaged DNA causes defective replication and may trigger apoptosis through the same mechanisms that induce cell death after constitutive expression of oncogenes. However, the activation of AP-1 in cells that are able to proliferate promotes cell proliferation and survival.
1.8.2 transcription factors p53

The p53 tumor suppressor gene can inhibit proliferation transiently, induce permanent cell-cycle arrest/senescence, or cause apoptosis depending on the cellular context. There is evidence for cross talk between the proliferation/differentiation pathways activated by Ras/Raf/MAPK and growth arrest functions of tumor suppressor genes including p53, p16, and Rb (Hollstein et al., 1991, Aless et al., 1995). The duration and intensity of MAPK activation can profoundly influence the biological response observed. Recent studies have shown that overexpression of Ras or Raf, which causes activation of MAPK, can induce p53 and/or p21-mediated growth arrest (Marshall et al., 1996). Furthermore, tumor suppressor p53 induction activates the MAPK cascade, while this function depends on p53 transcriptional activity, and MAPK activation by p53 is a consequence of Ras and Raf activation (Lee et al., 2000).

Normally, in a cell, the p53 protein is kept at a low concentration by its relatively short half-life. In addition to this low protein concentration, p53 probably also exists in a latent form, inactive for transcription in some cells. Under these conditions, the p53 protein must receive a signal or alteration to activate it to function. The upstream events or signals that flow to p53 are mediated by several stressful situations. Several different types of DNA damage can activate p53, including double-strand breaks in DNA by radiation or chemical damage to DNA. This results in a rapid increase in the level of p53 in the cell and activation of p53 as a transcription factor. The p53 level increases because the half-life of the protein is lengthened and possibly because the rate of translational initiation of p53 mRNA in the cell is enhanced. This increase in p53 levels is proportional to the extent of DNA damage, but both the extent of increase and the
kinetics of p53 enhancement differ for different types of radiation damage. The cell uses
different functions and proteins to recognize different classes of DNA damage (such as breaks in
the DNA and excision repair of ultraviolet-irradiation dimers) and different systems of enzymes
to repair them.

The downstream events mediated by p53 take place by two major pathways: cell cycle
arrest and apoptosis. p53 mediates a DNA damage checkpoint that relates to the
interrelationships among a number of oncogenes and tumor suppressor genes that regulate the
G1–S phase restriction point. This event decides the choice by p53 whether to initiate a G1
arrest (via p21) or apoptosis. In response to some forms of DNA damage, p53 is activated and
turns on the transcription of one of its downstream genes, p21(El-Deiry et al., 1993). p21 can act
on cyclin–Cdk complexes and proliferating cell nuclear antigen (PCNA) to stop DNA
replication.

More recently, p53 has been implicated in a G2/M phase checkpoint. In addition, p53
may be part of a G2/M checkpoint, preventing premature entry into another S phase. In addition,
p53 appears to be an integral part of the process that regulates the number of centrosomes in a
cell(Fukasawa et al., 1996)

p53 can also initiate apoptosis in response to the expression of a viral or cellular
oncogene or the absence of a critical tumor suppressor gene product (Rb). A number of factors
affect the decision of a cell to enter a p53-mediated cell cycle arrest or apoptotic pathway.
Under conditions in which the DNA is damaged, when survival factors for the cells are limiting,
or an activated oncogene (E1A, E7, E2F-1, or myc) is forcing the cell into a replicative cycle,
p53-mediated apoptosis prevails. In this way, cells with unstable genomes (due to DNA
damage) or cells in an abnormal environment (i.e., located in a place with limiting survival factors), with activated oncogenes that commit them to enter the cell cycle, are eliminated in a p53-dependent apoptotic event. This is most likely the reason why so many cancerous cells select against wild-type p53 function.

p53 protein can be detected by the western blotting technique in normal and neoplastic specimens of human endometrium. Treatment with GnRH agonist for 16 wk increased p53 protein content in leiomyomas (Gao et al., 2002). Cetrorelix, a GnRH antagonist, increased the protein levels of p21 and p53 and exerted a dose-dependent antiproliferative action on the growth of HTOA, a human epithelial ovarian cancer cell line (Tang et al., 2002)

1.9. Hypothesis

Collectively, these observations suggest that the role of GnRH in human reproduction should be re-evaluated.

As the presence of an immunoreactive GnRH in preimplantation embryos, and oviducts has been demonstrated, the GnRH system may be playing an important role during fertilization, preimplantation embryonic development, and the implantation process. Furthermore, GnRH I and GnRH II expression are also found in the human placenta and endometrium. These phenomenon gave rise to the idea that the GnRH system must exert distinct functions in the placenta and endometrium. On the other hand, proteinases including Matrix metalloproteinases (MMPs) and tissue specific inhibitor of matrix metalloproteinases (TIMPs), and the urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI) were suggested to play crucial roles in these processes.

Therefore, we have hypothesised that GnRH-1, GnRH-II and GnRHR play key regulatory role(s) in the cyclic remodeling events that occur in the endometrium in
preparation for pregnancy and/or in the formation and organization of the human placenta.

1.10 Specific aims

Specific aim 1: To investigate the effects of GnRH-I and GnRH-II on the urokinase-type plasminogen activator (UPA)/plasminogen activator inhibitor (PAI-1) system in human trophoblasts in vitro.

The regulated expression of the urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) is believed to modulate the invasive capacity of human trophoblastic cells in vitro and in vivo. To date, the factors capable of regulating the expression of uPA and PAI-1 in these cells remain poorly characterized. In these studies, we have examined the ability of the classical Gonadotropin-Releasing Hormone (GnRH I) and the second form of GnRH (GnRH II) to regulate uPA and PAI-1 mRNA and protein expression levels in primary cultures of human extravillous cytotrophoblasts using quantitative competitive PCR and ELISA, respectively.

Specific aim 2: To determine the different effects of GnRH I and GnRH II on the uPA/PAI-1 system in human decidual cells in vitro.

The establishment of a successful pregnancy is dependent upon the coordinated development of the implanting embryo and the maternal endometrium. In particular, the blastocyst must have attained the ability to attach to endometrial epithelium, and subsequently invade into the underlying decidua. However, unlike tumor cells, trophoblast invasion into the underlying maternal tissues is highly regulated and errors can have severe consequences for the health of both the mother and fetus. The terminal differentiation of the endometrial stroma into deciduas is believed to be a critical event in the development of a uterine environment that is capable of
fulfilling this embryo regulatory role. The urokinase plasminogen activator (uPA) and its endogenous inhibitor, plasminogen activator inhibitor (PAI)-1, play key roles in the highly regulated series of remodeling events that occur in the endometrium in preparation for pregnancy. To gain a better understanding of the role(s) of GnRH I and GnRH II in this developmental process, we have examined the ability of GnRH I and GnRH II to regulate uPA and PAI-1 mRNA and protein expression levels in primary cultures of stromal cells isolated from first trimester decidual tissues in a dose-and time-dependent manner.

Specific aim 3: To examine the regulatory effects of GnRH I and GnRH II on the levels of Matrix Metalloproteinase (MMP)-2, MMP-9, and tissue specific inhibitor of metalloproteinase-1 expression in primary cultures of human extravillous cytotrophoblasts.

An intricate balance between the production of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue specific inhibitors of matrix metalloproteinases (TIMPs), modulates the over-all proteolytic activity of trophoblasts during human implantation. In these studies, we have examined the ability of the classical Gonadotropin-Releasing Hormone (GnRH I) and the second form of GnRH (GnRH II) to regulate MMP-2, MMP-9 and TIMP-1 mRNA and protein levels in extravillous cytotrophoblasts propagated from first trimester chorionic villi.

Specific aim 4: To investigate the dose-dependent effects of GnRH on matrix metalloproteinase (MMP)-2, and MMP-9 and TIMP-1 mRNA levels in human decidual stromal cells in vitro.

Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue specific inhibitor of matrix metalloproteinases (TIMPs), play key roles in the cyclic remodeling events that occur in the human endometrium in preparation for pregnancy. To date, the factors capable of regulating
the expression of MMPs and TIMPs in the human decidua remain poorly characterized. The spatiotemporal expression of Gonadotropin Releasing Hormone (GnRH) in the human endometrium during the menstrual cycle and early pregnancy suggests that this hormone may have a regulatory role in the development of this dynamic tissue. In view of these observations, we have examined the ability of GnRH to regulate MMP-2, MMP-9 and TIMP-1 mRNA levels in primary cultures of human decidual stromal cells using a quantitative competitive polymerase chain reaction strategy.
PART II General Materials and Methods

2.1. Materials

All Chemicals reagents were purchased from SIGMA Chemical Co. (Oakville, ON, Canada) or Gibco-BRL Life Technologies (Burlington, ON, Canada). Cell culture media DMEM, supplemented with 4.5 mg/ml glucose, trypsin/EDTA solution (0.05% Trypsin, 0.53 mM EDTA), fetal bovine serum (FBS), 100 x Penicillin-Streptomycin-Glutamine solution and Gentamicin were purchased from Gibco (Burlington, ON, Canada). GnRH I or GnRH II were purchased from Peninsula Laboratories (Belmont, CA). The GnRH antagonist, Cetrorelix were purchased from AnaSpec, Inc. (San Jose, CA). Total RNA preparation kit, RNasey Mini Kit and first strand cDNA synthesis Kit were purchased from QIAGEN Inc. (Mississauga, Ontario, Canada) and Amersham-Pharmacia Biotech, Inc. (Morgan, ON, Canada). Phospho-specific antibodies used in these studies were purchased from New England Biolabs (Mississauga, ON, Canada). ELISA kits for MMPs, TIMP-1 uPA, PAI-1 or MAPK were purchased from American Diagnostica, Inc. (Greenwich, CA) or Biosource International Inc. (Camarillo, CA). Transfection reagents were purchased from Invitrogen life technologies (Carlsbad, CA), Luciferase Reporter Assays were purchased from Promega Corp. (Nepean, Canada). The p53-dependent or c-jun transcription activity kits were purchased from Activemotif Inc (Carlsbad, CA). Mercury pathway profiling systems were purchased from Clontech Inc. (Polo Alto, CA, USA). All oligodeoxynucleotides used in these studies were synthesized by the Oligonucleotide Synthesis Laboratory in University of British Columbia (Vancouver, BC, Canada). Consensus and mutated oligonucleotides for transcription factor binding sites and antibodies were purchased
from Santa Cruz biotechnology Inc. (Santa Cruz, California, USA) or Invitrogen Canada Inc. (Burlington, Ontario, Canada).

2.2. Tissue and cell isolation

Samples of first trimester placental tissues and decidual tissues were obtained from women undergoing elective termination of pregnancy (gestational ages ranging from 6–12 wk). The use of these tissues was approved by the Committee for Ethical Review of Research on the use of human subjects, University of British Columbia. All women provided informed written consent.

2.2.1 Trophoblast

Extravillous cytotrophoblasts (EVTs) were propagated from first trimester placental explants as described by Graham et al. Briefly, chorionic villi were washed thoroughly in DMEM (Life Technologies, Inc., Burlington, Ontario, Canada) containing antibiotics. The villi were minced finely and plated in 25-cm\(^2\) tissue culture flasks containing DMEM supplemented with antibiotics and 10% heated-inactivated fetal bovine serum (FBS). The fragments of chorionic villi were allowed to adhere for 2–3 d, after which the nonadherent material was removed. The villous explants were cultured for a further 10–14 d with the culture medium being replaced every 48 h. The EVTs were separated from the villous explants by a brief (2–3 min) trypsin digestion [0.125% (vol/vol) trypsin-EDTA/Ca\(^{2+}\), Mg\(^{2+}\)-free PBS] at 37 \(^\circ\)C and plated in 60-mm culture dishes (Falcon, Becton Dickinson and Co. Labware, Franklin Lakes, NJ) penicillin/streptomycin (100 IU/ml, 100 \(\mu\)g/ml, respectively) and supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The purity of the EVT cultures was determined by immunostaining with a monoclonal antibody directed against cytokeratin 8 and 18 (Becton Dickinson and Co.) according to the methods of MacCalman et al.. Only cell cultures that exhibited 100% immunostaining for cytokertain were included in these studies.
2.2.2 Decidual stromal cells

Stromal cells were isolated from the decidual tissue samples by enzymatic digestion and mechanical dissociation using a protocol modified from that reported by Shiokawa et al. Briefly, the decidual tissue samples were minced and subjected to 0.1% collagenase (type IV, Sigma, St. Louis, MO) and 0.1% hyaluronidase (type I-S) digestion in a shaking water bath at 37°C for 60 min. The cell digest was then passed through a nylon sieve (38 μm). The isolated glands and any undigested tissue fragments were retained on the sieve, and the elute containing the stromal cells was collected in a 50-ml tube. The stromal cells were then pelleted by centrifugation at 800 x g for 10 min at room temperature. The cell pellet was washed once with DMEM containing 10% fetal bovine serum (FBS) before being resuspended and plated in DMEM containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% FBS. The culture medium was replaced 30 min after plating to reduce epithelial cell contamination. The purity of the decidual stromal cell cultures was determined by immunocytochemical staining for vimentin, cytokeratin, muscle actin, and factor VIII (data not shown). These cellular markers have been used to determine the purity of human endometrial cell cultures (Irwin JC et al 1989). As defined by these criteria, the decidual stromal cell cultures used in these studies contained less than 1% epithelial or vascular cells.

2.3. Treatment

3.1. To determine the effects of GnRH I or GnRH II on uPA and PAI-1 or MMP-2, MMP-9 and TIMP-1 mRNA and protein levels in EVTs or decidual stromal cells, cells were cultured in the presence or absence of a fixed concentration (100nM) of GnRH I or GnRH II (Peninsula Laboratories Inc, Belmont, CA) for 0, 3, 6, 12, 24 or 48 h or increasing concentrations of these two hormones (0, 0.1, 1, 10, or100nM) for 24 h. In addition, EVT or decidual stromal cell cultures were treated with GnRH I or GnRHII (100nM) in combination
with increasing concentrations of the GnRH antagonist, Antide or Cetrorelix (AnaSpec Inc., San Jose, CA), (1nM, 10nM, or 100nM) for 24h. EVT cultures treated with vehicle alone served as control for these experiments.

The cells were harvested for RNA extraction and the conditioned culture medium collected for ELISA.

2.4. Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) amplification

2.4.1 Total RNA Isolation and RT

Total RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen, Inc, CA) according to the manufacturer’s suggested procedure. The RNA concentration was quantified by measuring the optical density with a Du-64 UV-spectrophotometer (Beckman Coulter).

RNA obtained from human EVTs or decidual stromal cells was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Oakville, Canada). One microgram of total RNA dissolved in DEPC-water (8 μl in total) was heated at 65°C for 10 min and cooled on ice for 5 min. DTT (1 μl), oligo-dT (1 μl) and bulk mixture (5 μl) was added to the sample, and the mixture was incubated at 37°C for 1h. After incubation, the sample was boiled for 10 min to inactivate reverse transcriptase and subsequently stored at −20°C until use.

The concentration and purity of RNA were determined based on absorbance at 260 nm measured by a spectrophotometer (Model DU-64, Beckman). cDNA was synthesized from total RNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Oakville, Canada). The reaction mixture (15μl), containing 1 μg
RNA, 5 μl bulk first-strand reaction mix, 0.2 μg oligo-dT primer and 6 mM dithiothreitol (DTT), was incubated at 37°C for 60 min and terminated by heating at 90°C for 5 min.

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

2.4.2 Primer design

Primers specific for human GnRH I, GnRH II, GnRH receptor, uPA, PAI-1, MMP-2, MMP-9, TIMP-1 and GAPDH were designed. Nucleotide sequences specific to those genes, which also spanned different exons, were identified in the human mRNA sequences (Table 1.) deposited in GenBank (National Center for Biotechnology Information). Forward and reverse primers corresponding to these DNA sequences, were synthesized at the NAPS Unit, University of British Columbia. The primer sequences and the expected sizes of the resultant PCR products are listed in Table.

2.4.3 Polymerase Chain Reaction (PCR)

PCR was performed using template cDNA generated from the total RNA extracts prepared from the cultured EVTs and decidual stromal cells. Semiquantitative PCR was performed using template cDNA generated from the total RNA extracts prepared from cultures of untreated decidual stromal cells and the primers specific for MMP-2, MMP-9, or TIMP-1. The PCR conditions were as follows: 1 min at 94 C, 1 min at 56 C, 59 C and 56 C for MMP-2,
MMP-9, or TIMP-1 or 56 C and 57.5 C for PAI-1 and u-PA, respectively; and 1.5 min at 72 C followed by a final extension at 72 C for 15 min. The cycles were repeated 20–35 times.

The resultant PCR products of 505, 544, and 369 bp for MMP-2, MMP-9, and TIMP-1, and 687, and 622 bp for PAI-1 and uPA, respectively, were separated using gel electrophoresis and visualized by ethidium bromide staining. To confirm the specificity of the primers, an aliquot of the MMP-2, MMP-9, TIMP-1, PAI-1 and uPA cDNAs were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequence analysis.

2.5. Quantitative competitive (QC)-PCR

The competitive reverse primers based on the same nucleotide sequence as primers, but in which an additional stretch of base pairs, corresponding to short nucleotide sequence identified in the target cDNAs upstream of the binding region of the original reverse primers, were incorporated into their 5' ends (Fig. 8).

To determine the equivalence of target cDNA and internal standard cDNA, serial dilutions of the internal standard cDNA for MMP-2, MMP-9, TIMP-1, uPA and PAI-1, were coamplified in the presence of target cDNA. The point at which the two graphs cross indicated the amount of internal standard cDNA that should be added. One picogram of competitive cDNA was added to each unknown sample before QC-PCR according to the equivalences The standard curve for MMP-2, MMP-9, TIMP-1, uPA and PAI-1 was constructed by coamplification of a fixed amount of mutant cDNA and the varying concentration of the native cDNA obtained by serial dilutions. One microliter each of the mutant and native cDNA mix were added to 23 μl PCR-Mastermix containing 1.5 mmol/liter MgCl₂ solution, 10x PCR buffer minus Mg²⁺, 0.2 mmol/liter of each deoxy-nucleoside 5'-triphosphate, 2.5 U Taq polymerase (all from Life Technologies, Inc.), and their corresponding paired primers (2 μmol/liter) to give a
The PCR conditions and number of cycles were performed for the MMP-2, MMP-9, TIMP-1, uPA and PAI-1 primer sets as described above. An aliquot (10 μl) of the PCR products was separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide. The intensity of the ethidium bromide staining of the PCR products was analyzed using UV densitometry (Biometra, Whiteman Co., Gottingen, Germany). Volume counts (square millimeters) of the PCR products were then determined using the Scion Image software (Scion Image Corp., Frederick, MD). MMP-2, MMP-9, TIMP-1, uPA and PAI-1 standard curves were then generated using the logarithmically transformed ratios of target:competitive cDNA plotted against the log amount of target cDNA included in the PCR reactions. These standard curves were highly reproducible and linear. The values obtained from this regression curve were then used to determine the amounts of MMP-2, MMP-9, TIMP-1, uPA and PAI-1 transcripts present in unknown samples.

2.6. Southern Blot Analysis

The membrane was hybridized and detected following the manufacturer's recommended procedures with a digoxigenin (DIG)-dUTP-labeled GnRH I, GnRH II and GAPDH cDNA probe (Boehringer Mannheim, Laval, Canada). Hybridization was performed at 42°C overnight in the presence of 5 x SSC (75 mM sodium citrate, 0.75 M NaCl, pH 7.0), 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS, 1% w/v blocking reagent and 50% formamide. The membrane was then washed twice with 2 x SSC and 0.1% SDS at room temperature, and followed by twice with 0.1 x SSC and 0.1% SDS at 68°C. After high stringency washing, the membrane was rinsed briefly in washing buffer (maleic acid buffer plus 0.3% Tween-20), and incubated with 1% blocking reagent in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 30 min. An anti-DIG conjugated alkaline phosphatase (diluted in 1:10000 in washing buffer) was added and incubated for addition 30 min. The membrane was then washed twice.
with washing buffer and equilibrated for 3 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl$_2$, pH 9.5). Chemiluminiscent reaction was started by the addition of CSPD (1:100), and exposed to Kodak Omat X-ray film. The radioautograms were scanned and quantified with Scion Image-Released Beta 3b (Scion Corporation, Maryland, USA). The relative GnRH I and GnRH II mRNA level was calculated by normalizing the PCR products for GnRH I,GnRH II against the GAPDH expression.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of MMP-2, MMP-9, TIMP-1, uPA and PAI-1 in conditioned medium were measured by ELISA. uPA (Chemicon International, Inc., Temecula, CA) was detected in the conditioned culture medium with a mean intra-assay and inter-assay coefficient of variation of 4.9% and 8.2%, respectively. PAI-1 (American Diagnostica, Inc., Greenwich, CT) was detected in the conditioned medium with a mean intraassay and interassay coefficient of variation of 6.1% and 8.8%, respectively. All samples were assayed in duplicate.

2.8. Molecular Cloning and Sequencing

The PCR products were separated by gel electrophoresis. The DNA was excised and purified from the agarose by hot-phenol method with modification (Falson 1995). Briefly, the excised DNA in agarose gel was re-melted by heating at boiling water for 5 min by adding 1 x TBE containing 0.5 M NaCl. After melting, the mixture was further incubated at 65°C for 10 min. Then, an equal volume of preheated (65°C) phenol was added to the melted gel and mixed. The aqueous phase was separated by centrifugation (14000 rpm for 10 min at 4°C), re-extracted with phenol/chloroform followed by chloroform/isoamyl alcohol, and precipitated with ethanol.
The purified PCR products was ligated into a PCR II vector (Invitrogen, Carlsbad, CA, USA) at 14°C overnight and transformed into their host bacteria.

2.9. Transient transfections

Transient transfections were performed using Lipofetamine™ 2000 transfection reagent (Invitrogen life technologies Carlsbad, CA), following the manufacturer’s protocol. To correct for different transfection efficiencies of various luciferase constructs, the Rous sarcoma virus (RSV)-lacZ plasmid was cotransfected into the cells with 1µg vectors that contained a specific cis-acting DNA sequence (enhancer element) and a sensitive luciferase reporter gene. Briefly, 4 x 10^5 of cells were seeded into six-well tissue culture plates before the day of transfection. One microgram of the various luciferase reporter constructs and 1 µg of RSV-lacZ vector were cotransfected into the cells under serum-free conditions. After 6 h of transfection, 1 ml of medium containing 20% FBS was added, and the cells were further incubated for 24 h. After incubation, the old medium was removed and the cells were cultured for another 24 h with normal fresh medium containing 10% FBS. The treatments were carried out 40 hours after transfection and the cells were exposed in the presence of 100nM GnRH I or II for 6 hr.

2.10 Luciferase Reporter Assays

Cellular lysates were collected with 150µl reporter lysis buffer (Promega Corp., Nepean, Canada) and immediately assayed for luciferase activity with the Luciferase Assay System (Promega Corp. Nepean, Canada) at room temperature. The transfected cells were rinsed twice with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.47 M KH2PO4) and incubated for 15 min after addition of 200 µl of cell lysis buffer. Cells were then collected by centrifugation at 14000 rpm for 10 s at 4°C. Fifty µl of cell lysates was mixed with 100 µl each of Substrate A and Substrate B, and luminescence was immediately measured by
Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Beta-galactosidase (β-Gal) activity was also measured and used to normalize for varying transfection efficiencies. Promoter activity was calculated as luciferase activity/β-galactosidase activity. A promoterless pGL2-Basic vector was included as a control in the transfection experiments.

2.11 Plasmid

Pp53-TA-Luc vector (Clontech, Polo Alto, CA, USA) is designed to monitor the induction of p53 signal transduction pathway and contains the firefly luciferase (luc) gene from Photinus pyralis. pp53-TA-Luc contains a p53 response element, located upstream of the minimal TA promoter, the TATA box from the herpes simplex virus thymidine kinase promoter (P_{TA}). Located downstream of P_{TA} is the firefly luciferase reporter gene (luc). After transcription factors bind to the p53-response elements, transcription is induced and the reporter gene is activated. pTAL-Luc (Clontech) was used as a negative control to determine the background signals associated with the cell lysates. The enhancerless pTAL-Luc contains HSV-TK upstream of the Luciferase coding sequence.

2.12 Microspectrofluorimetry

Trophoblasts and decidual cells were seeded onto 25-mm circular glass cover slips (5000 cells/slip) and incubated for 3 days at 37°C in humidified air with 5% CO₂ before microfluorimetric experiments. Cytosolic calcium concentrations were measured using the dural-excitation single-emission fluorimetric technique. Briefly, the cells were incubated with 5-10 μM fura-2 AM acetoxymethyl ester (Molecular Probes, Inc) for 30 min at 37°C in humidified air with 5% CO₂. The coverslip was mounted onto the perifusion chamber and equilibrated for 10 min with balanced salt buffer in humidified air with 5% CO₂. The fura-2 ratio measurements
were performed using the Attoflour Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). The perifusion chamber was connected to a multiunit six-channel perfusion system with a flow rate of 1-2 mL/min. Fura-2-loaded cells were observed through a ×40 fluorescent objective lens and were illuminated alternatively with light at 340 nm and 380 nm. Emitted light was filtered using a 510-nm long pass filter and detected using a low light-sensitive camera. Measurements of cytosolic calcium were performed at 1- to 2-sec intervals. All records were corrected for background fluorescence ratio (determined from cell-free region of coverslip). Changes in the fluorescence ratio recorded at 340 and 380 nm correspond to changes in cytosolic calcium.

Trophoblastic and decidual cells were treated with various concentrations of GnRH I and GnRH II for dose-response experiments. PGF$_2$α treatment was used as a positive control.

2.13 Preparation of nuclear extracts

Nuclear extracts were prepared from human EVTs or decidual stromal cells according to the methods described previously (Lassar et al. 1991). Briefly, cells at 70% confluency will be lysed in 2ml of Lysis Buffer, containing 20mM HEPES pH 7.6, 20% Glycerol, 10mM NaCl, 1.5mM MgCl$_2$, 0.2mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 100 µg/ml aprotinin. Cells were dislodged by scrapping and collected by centrifugation for 5 min at 2000 rpm at 4°C. Nuclei were resuspended at 2.5 x 10$^7$ nucleus per ml in Nuclear Extraction Buffer (Lysis Buffer plus 500mM NaCl). Nuclei were gently rocked for 1h at 4°C and centrifuged at 10,000 rpm for 10 min, and supernatant was aliquoted, quick frozen in liquid nitrogen, and stored at -80°C.
2.14 ERK1/2 activity assay

The levels of activated ERK ½ were determined by solid phase sandwich enzyme link immuno-sorbent assay (ELISA). ERK ½ activity assay were carried out as described by the protocol of Biosource International Inc. In brief, decidual stromal cells were starved for 3hrs in FBS free DMEM at 37°C, 5% CO₂ atmosphere incubator. The cells were washed once with phosphate-buffered saline and the treatments with GnRH I or II were carried out for the amount of dose or time indicated in each figure before harvest. The cells were then washed with PBS and lysed in 200μl of cell extraction buffer ( 10 mM Tris, (pH 7.4), 100 mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na₄P₂O₇, Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5%deoxycholate, 1mM phenylmethylsulfonyl fluoride, Protease inhibitor cocktail). The activity of ERK ½ in the cell lysate was measured by ELISA. A monoclonal antibody specific for ERK ½ (regardless of phosphorylation state) was coated onto the wells of microtiter strips provided. The samples and controls were pipetted into these wells. During the first incubation, the ERK ½ antigens bind to the immobilized antibody. After washing, an antibody specific for ERK ½ phosphorylated at threonine 185 and tyrosine 187 is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized ERK ½ proteins captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG was added. This binds to the detection antibody to complete the four member sandwich. After a third incubation and washing to remove all the excess antirabbit IgG-HRP, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The absorbance of each well was determined at 450 nm with a MRX microplate reader ( DYNEX Technologies, Chantilly, VA). ERK ½ was detected by ELISA with a mean intra-assay and inter-assay coefficient of variation of 5.7% and 6.4%, respectively. All samples were assayed in duplicate.
2.15 P38 MAPK activity assay

The activity of p38MAPK in the cell lysate was measured by ELISA. The p38 kinase assay was carried out as described by the manufacturer (Biosource International Inc, Camarillo, CA). The cell preparation and treatment are similar as described in the ERK assay above. Then, the cells were washed once with ice-cold phosphate-buffered saline and lysed in 200 µl of lysis buffer per sample. The lysate samples and controls were pipetted into these wells. An antibody specific for p38 MAPK phosphorylated at threonine 180 and tyrosine 182 was added to the wells. The rest of the procedure was as described above. p38 MAPK was detected by ELISA with a mean intra-assay and inter-assay coefficient of variation of 4.3% and 8.4%, respectively. All samples were assayed in duplicate.

2.16 EMSA (electrophoretic mobility shift assay)

Oligonucleotides containing the consensus p53 binding sequence (5'-CTTGGACATGCCCCGGGATGCTCCCTC-3') was purchased from Invitrogen and annealed to form double-strand DNA. Probes for EMSAs were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase and separated from unincorporated radionucleotides by G-50 Micro Columns (Amersham Pharmacia Biotech). Protein concentrations were determined by a modified Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). EMSAs were carried out in 20 µl containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 10 % glycerol, 1 µg poly (dI:dC), 20 µg nuclear proteins and 50 fmol radiolabeled probe (30,000 cpm). For the competitive assays, competitor oligonucleotides were added simultaneously with the radiolabeled probes. The binding reaction was incubated at room temperature for 30 min and then separated by a 5 % PAGE gel containing 0.5 x TBE (0.09 M
tris-borate and 2 mM EDTA, pH 8.0) at constant 200 V and at 4 C. Before loading of samples, the gel was pre-run for 90 min at 100 V. After electrophoresis, the gel was dried and exposed to a Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at -70 C.

2.17 Western Blot analysis

Cells were treated with GnRH I or GnRH II in the presence or absence 100 nM Cetrorelix, 10μM PD 98059, 10μM SB203580 for 6 hours. Cells were lysed in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 50 μM leupeptin). Lysates were centrifuged for 10 min at 14,000 rpm in an Eppendorf microcentrifuge; 90 μl of the supernatants were mixed with 30 μl of 4×Laemmli sample buffer. Samples were boiled for 5 min. Twenty μl of the prepared samples were electrophoresed through a 12% SDS-polyacrylamide gel and the separated proteins were then electrophoretically transferred onto nitrocellular paper (Hybond-C, Amersham-Pharmacia Biotech, Morgan, Canada). Membranes were incubated in blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 2% bovine serum albumin, and 0.1% NaN3) at 4 °C overnight; then monoclonal rabbit anti-phospho-p53 (Ser 20) antibody or anti-total p53 antibody (New England Lab 1:1000) was added to the blocking buffer, and blots were incubated for an additional 2 h at room temperature. The blots were washed three times in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1 % Tween 20) and incubated with Horseradish Peroxidase(HRP)-conjugated anti-rabbit Ig (Amersham; 1:2,000 in TBST) for 1 h at room temperature. The blots were washed three times in TBST. The Amersham enhanced chemiluminescence system (ECL) was used for detection. Membranes were visualized by exposure to Kodak X-Omat film. The radioautograms
were then scanned and quantified with Scion Image-Released Beta 3b software (Scion Corporation, Maryland, USA).

2.18 Immunoprecipitation Assay

The level of p53 phosphorylation at serine 20 induced by GnRHs and the complex of p53 with p38 kinase was measured by Western blot for immunoprecipitation using specific antibodies against p53. Briefly, decidual stromal cells were cultured in 100-mm dishes with 10% FBS DMEM until they reached 80% confluence. Then, the cells were starved by culturing them in 1% FBS MEM for 12 h. The cells were exposed to GnRH for induction of p53 phosphorylation at serine 20. The cells were lysed on ice for 1 h in the lysis buffer and spun at 14,000 rpm for 5 min. The lysates were immunoprecipitated using p53 antibodies (Ab1) and protein G agarose. The bands were washed, and the phosphorylated protein of p53 at serine 392 and the p38 kinase, as well as phosphorylated p38 kinase, were selectively measured by Western immunoblotting using a specific antibody and chemiluminescent detection system.

2.19 P53 transcription factor assay

p53-dependent transcription activity was assayed by using nuclear extract from primary cultures of decidual stromal cells. Confluent monolayers of decidual stromal cells were cultured in 100 mm culture plates containing 10% FBS DMEM. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells (100-mm dishes) were serum deprived for 16 h before treatment. The cells were treated with GnRH I or II and different concentrations of inhibitors for 6 h. Nuclear extracts were prepared from these decidual stromal cells. The p53-dependent transcription activity was measured by Active Motif's TransAM™ kits (Activemotif Inc, Carlsbad, CA) which are ELISA-based assays for the detection of transcription factor activation.
in mammalian tissue and cell extracts. In brief, the cell extracts containing activated transcription factors were added into a 96-well plate on which has been immobilized a cis-response element oligonucleotide containing a p53 consensus binding site (5'-GGACATGCCCCGGCATGTCC-3'). Anti-p53 antibody, which recognizes accessible epitopes on c-jun proteins upon DNA binding, were added and incubated for 1 h at room temperature. Anti-rabbit HRP-conjugated antibody was added to all wells being used. The colorimetric readout was quantified by MRX microplate reader (Dynex Technologies, Inc. Chantilly, VA) under colorimetric reaction. The results are expressed as relative p53 activity.

2.20 C-JUN transcription factor assay

The nuclear extracts were prepared from cultured cells. The c-jun-dependent transcription activity was measured by Active Motif's TransAM™ kits (Activemotif Inc, Carlsbad, CA). In brief, the cell extracts containing activated transcription factors were added into a 96-well plate on which has been immobilized a cis-response element oligonucleotide containing a AP-1 consensus binding site (5'-TGAGCTCA-3'). Anti-c-jun antibody, which recognizes accessible epitopes on c-jun proteins upon DNA binding, were added and incubated for 1 h at room temperature. Anti-rabbit HRP-conjugated antibody was added to all wells being used. The colorimetric readout was quantified by MRX microplate reader (Dynex Technologies, Inc. Chantilly, VA) under colorimetric reaction. The results are expressed as relative c-jun activity.

2.21 Data analysis

All data were shown as the means +/- SD of triplicate assays in at least three independent experiments. For Western blot analysis, data are obtained from three independent experiments. All data were analyzed by one-way ANOVA followed by Tukey's multiple
comparison test or *t*-test using the computer software PRISM GraphPad Version 2 (GraphPad Software, Inc., San Diego, USA). Data were considered significantly different from each other when $P < 0.05$. 
# TABLE 1. Oligonucleotide primers for mRNA amplification

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<th>Primers 5'-3'</th>
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PART III. Effects of GnRH I and GnRH II on the uPA /PAI System in Human

Extravillous Cytotrophoblasts

3.1 Abstract

The regulated expression of the urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) is believed to modulate the invasive capacity of human trophoblastic cells \textit{in vitro} and \textit{in vivo}. To date, the factors capable of regulating the expression of uPA and PAI-1 in these cells remain poorly characterized. In these studies, we have examined the ability of the classical mammalian GnRH I and the second form of GnRH (GnRH II) to regulate uPA and PAI-1 mRNA and protein expression levels in primary cultures of human extravillous cytotrophoblasts using quantitative competitive PCR and ELISA, respectively. Both GnRH I and II increased uPA and concomitantly decreased PAI-1 mRNA and protein expression levels in our extravillous cytotrophoblast cultures in a dose- and time-dependent manner. Cetrorelix, a peptide GnRH antagonist specific for the GnRH I receptor, was capable of inhibiting the regulatory effects of GnRH I, but not GnRH II on uPA and PAI-1 expression levels in primary cell cultures. Taken together, these observations suggest that GnRH I and GnRH II may facilitate trophoblast invasion by increasing the ratio of uPA/PAI-1 expression via interactions with two distinct GnRH receptors.
3.2 Introduction

In the human and higher primates, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI-1) have been shown to be spatiotemporally expressed at the maternal-fetal interface during the first trimester of pregnancy (Uszynski et al., 2001. Hu et al., 1999). In particular, uPA and PAI-1 have been detected in the subpopulation(s) of extravillous cytotrophoblasts that invade deeply into the decidual tissues and uterine arterioles, thereby ensuring a continuous blood supply to the placenta (Fisher et al., 1993). PAI-1 increases steadily during pregnancy (Houlihan et al., 1996). The production of uPA by human trophoblasts is down-regulated during the second trimester, paralleling the decline in the invasiveness of these cells with gestational age. Taken together, these observations suggest that the balance between uPA and PAI-1 expression regulate, at least in part, the invasive capacity of human extravillous cytotrophoblasts. However, unlike tumor cell invasion, trophoblast invasion into the underlying maternal tissues is highly regulated (Lala et al., 2002). To date, the factors capable of regulating uPA/PAI-1 expression in highly invasive extravillous cytrophoblasts remain poorly characterized.

GnRH is a decapeptide best known for its role in regulating the release of gonadotropins from the pituitary. However, there is increasing evidence to suggest that in addition to this classical
pathway, GnRH may have direct regulatory actions on the development and function of the
gonads and other reproductive tissues, particularly the endometrium and placenta (Siler-Khodr et al., 1979, Pahwa et al., 1991). Furthermore, recent studies have demonstrated that a distinct
gene encoding a second form of GnRH, termed GnRH II, to distinguish it from the classical
mammalian form (GnRH I), is expressed in the extrapituitary tissues of the human and other
primates (White et al., 1998, Neil et al., 2001, Millar et al., 2001). To date, the biological
function of GnRH II in the human is not known.

GnRH analogs have been shown to be capable of down-regulating the invasive capacity of
breast, prostate, and uterine carcinoma cells and benign endometriotic cells in vivo and in vitro
(Grundker et al., 2002). These regulatory effects are believed to be mediated by the differential
expression of matrix metalloproteases (MMPs), their tissue-specific inhibitors (TIMPs), uPA,
and/or PAI-1. As the levels of GnRH I in the human placenta progressively increase during the
first 24 wk of gestation (Siler-Khodr et al., 1978), it is tempting to speculate that the invasive
capacity of extravillous cytotrophoblasts may be regulated, at least in part, by the
GnRH-mediated expression of uPA/PAI-1 in these cells.
3.3 Materials and Methods

3.3.1 Tissues and cell isolation

Samples of first trimester placental tissues were obtained from women undergoing elective termination of pregnancy (gestational ages ranging from 6–12 wk). The use of these tissues was approved by the Committee for Ethical Review of Research on the use of human subjects, University of British Columbia. All women provided informed written consent.

Extravillous cytotrophoblasts (EVTs) were propagated from first trimester placental explants as described by Graham et al. (Graham et al., 1992). Briefly, chorionic villi were washed thoroughly in DMEM (Life Technologies, Inc., Burlington, Ontario, Canada) containing antibiotics. The villi were minced finely and plated in 25-cm² tissue culture flasks containing DMEM supplemented with antibiotics and 10% heated-inactivated FBS. The fragments of chorionic villi were allowed to adhere for 2–3 d, after which the nonadherent material was removed. The villous explants were cultured for a further 10–14 d with the culture medium being replaced every 48 h. The EVTs were separated from the villous explants by a brief (2–3 min) trypsin digestion [0.125% (vol/vol) trypsin-EDTA/Ca²⁺, Mg²⁺-free PBS] at 37 C and plated in 60-mm culture dishes (Falcon, Becton Dickinson and Co. Labware, Franklin Lakes, NJ) penicillin/streptomycin (100 IU/ml, 100 μg/ml, respectively) and supplemented with 10% fetal
bovine serum (Life Technologies, Inc.). The purity of the EVT cultures was determined by immunostaining with a monoclonal antibody directed against cytokeratin 8 and 18 (Becton Dickinson and Co.) according to the methods of MacCalman et al. (MacCalman et al., 1996). Only cell cultures that exhibited 100% immunostaining for cytokertain were included in these studies.

All studies were performed using EVTs (passage 2) plated in 60-mm culture plates at a density of 1 x 10^6 cells (Falcon, Becton Dickinson and Co.). Twenty-four hours before each treatment, serum was removed from the culture medium.

3.3.2 Cell treatments

To determine the effects of GnRH I or GnRH II on uPA and PAI-1 mRNA and protein levels in EVTs, cells were cultured in the presence or absence of a fixed concentration (100 nM) of GnRHI or GnRH II (Peninsula Laboratories, Inc., Belmont, CA) for 0, 3, 6, 12, 24, or 48 h or increasing concentrations of these two hormones (0, 0.1, 1, 10, or100 nM) for 24 h. The concentration of GnRH I and GnRH II used in these experiments were selected on the basis of previous studies (Raga et al., 1999). In addition, EVT cultures were treated with GnRH I or GnRH II (100 nM) in combination with increasing concentrations of the peptide GnRH
antagonist, Cetrorelix (AnaSpec, Inc., San Jose, CA), (1, 10, or 100 nM) for 24 h. EVT cultures treated with vehicle alone served as control for these experiments.

The cells were harvested for RNA extraction and the conditioned culture medium collected for ELISA.

3.3.3 Generation of first-strand cDNA

Total RNA was prepared from the EVT cultures using a RNeasy Mini Kit (QIAGEN, Valencia, CA) using the protocol recommended by the manufacturer. The concentration of total RNA present in each of the extracts was quantified by optical densitometry (260/280 nm) using a Du-64 UV-spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

An aliquot (1 µg) of the total RNA extracts prepared from these EVT cultures was reverse transcribed into cDNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Oakville, Canada).

3.3.4 Primer design

Two sets of primers specific for human uPA or PAI-1 were designed. Nucleotide sequences specific to human uPA and PAI-1, which also spanned different exons, were identified in the human mRNA sequences (See Table) deposited in GenBank (National Center for Biotechnology
Information. Forward and reverse primers corresponding to these DNA sequences (primers 1 and 2 for uPA and primers 4 and 5 for PAI-1, respectively), were synthesized at the NAPS Unit, University of British Columbia. A set of competitive reverse primers (primers 3 and 6 for uPA and PAI-1, respectively) based on the same nucleotide sequence as primers 2 and 5, but in which an additional stretch of base pairs, corresponding to short nucleotide sequence identified in the target cDNAs upstream of the binding region of the original reverse primers, were incorporated into their 5' ends (Fig. 8). Primers specific for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have been previously described.

### 3.3.5 PCR

PCR was performed using template cDNA generated from the total RNA extracts prepared from the cultured EVTs and combinations of the uPA and PAI-1 specific primers. The PCR conditions were as follows: 1 min at 94 C, 1 min at 57.5 C or 56 C for PAI-1 and uPA, respectively; and 1.5 min at 72 C followed by a final extension at 72 C for 15 min. The cycles were repeated 20–35 times.

A combination of primers 1 and 2 yielded the expected PCR product of 622 bp, corresponding to uPA, whereas primers 1 and 3 generated a truncated, competitive uPA cDNA of 419 bp. Similarly, a combination of primers 4 and 5 or 4 and 6 generated uPA cDNAs of 687 bp and 464,
respectively. The resultant PCR products were subcloned into the PCR II vector and subjected to DNA sequence analysis to confirm the specificity of the primers. A linear relationship between the number of PCR cycles and yield of PCR product was observed after 21 cycles for GAPDH, 27 cycles for uPA and 30 cycles for PAI-1 (data not shown).

3.3.6 Quantitative competitive (QC)-PCR

To determine the equivalence of target cDNA and internal standard cDNA, serial dilutions of the internal standard cDNA for uPA and PAI-1 were coamplified in the presence of target cDNA. The point at which the two graphs cross indicated the amount of internal standard cDNA that should be added. One picogram of uPA or PAI-1 competitive cDNA was added to each unknown sample before QC-PCR according to the equivalences (Fig. 9, A and B).

The standard curve for uPA and PAI-1 was constructed by coamplification of a fixed amount of mutant cDNA and the varying concentration of the native cDNA obtained by serial dilutions. One microliter each of the mutant and native cDNA mix were added to 23 μl PCR-Mastermix containing 1.5 mmol/liter MgCl₂ solution, 10x PCR buffer minus Mg²⁺, 0.2 mmol/liter of each deoxy-nucleoside 5'-triphosphate, 2.5 U Taq polymerase (all from Life Technologies, Inc.), and their corresponding paired primers (2 μmol/liter) to give a total volume 25 μl. The PCR conditions and number of cycles were performed for the uPA and PAI primer sets as described
above. An aliquot (10 μl) of the PCR products was separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide. The intensity of the ethidium bromide staining of the PCR products was analyzed using UV densitometry (Biometra, Whiteman Co., Gottingen, Germany). Volume counts (square millimeters) of the PCR products were then determined using the Scion Image software (Scion Image Corp., Frederick, MD). Two standard curves were then generated using the logarithmically transformed ratios of target:competitive cDNA plotted against the log amount of target cDNA included in the PCR reactions (Fig. 9, C and D). These standard curves were highly reproducible and linear. The values obtained from this regression curve were then used to determine the amounts of uPA or PAI-1 transcripts present in unknown samples.

An aliquot (1 pg) of either uPA or PAI-1 competitive cDNA was added to each unknown sample before QC-PCR was performed using the corresponding primer sets and PCR conditions described above. The ratios of the densities of sample target cDNA bands (622 and 687 bp) to competitive cDNA (419 and 464 bp) were normalized by GAPDH, logarithmically transformed and compared with the values obtained from the standard curve.

3.3.7 ELISA

The levels of uPA and PAI-1 in conditioned medium were measured by ELISA. uPA (Chemicon
International, Inc., Temecula, CA) was detected in the conditioned culture medium with a mean intraassay and interassay coefficient of variation of 4.9% and 8.2%, respectively. PAI-1 (American Diagnostica, Inc., Greenwich, CT) was detected in the conditioned medium with a mean intraassay and interassay coefficient of variation of 6.1% and 8.8%, respectively. All samples were assayed in duplicate.

3.3.8 Statistical analysis

The absorbance values obtained from the ethidium bromide-stained gels were subjected to statistical analysis using GraphPad Software, Inc. Prism 2 software (San Diego, CA). Statistical differences between the absorbance values were assessed by the ANOVA. Differences were considered significant for $P < 0.05$. Significant differences between the means were determined using Dunnett’s test. The results are presented as the mean relative absorbance ($\pm$SEM) obtained using five or more different tissue samples.

3.4. Results

3.4.1 Time effects of GnRH I or GnRH II on uPA and PAI-1 mRNA levels in cultured EVT

uPA and PAI-1 mRNA transcripts were detected in all of the total RNA extracts prepared from the EVT cultures. The addition of vehicle alone had no significant effect on uPA and PAI-1
mRNA levels in cultured EVTS at any of the time points examined in these studies (data not shown).

There was a significant increase in uPA mRNA levels in EVTs with time in culture in the presence of either GnRH I or GnRH II, with maximum levels being observed after 12 h (Fig. 10, A and B). There was a subsequent decline in levels of the uPA mRNA transcripts in the EVTs treated with GnRH I. However, the levels were still significantly greater than those observed in the 0 h control (Fig. 10A). In contrast, the levels of the uPA mRNA transcript in EVTs cultured in the presence of GnRH II remained relatively constant until the termination of these studies at 48 h (Fig. 10 B).

A significant decrease in PAI-1 mRNA levels was observed in cells cultured in the presence of GnRH II for 3 h (Fig. 10 D). A decline in the levels of PAI-1 mRNA transcripts in EVTs cultured in the presence of GnRH I was not observed until 24 h (Fig. 10 C). The PAI-1 mRNA levels in these cultures continued to decrease until the termination of these studies at 48 h (Fig. 10, C and D).

3.4.2 Effects of GnRH I and GnRH II of uPA mRNA and protein expression levels in cultured EVTs

GnRH I and GnRH II were capable of increasing uPA mRNA levels in EVTs in a
dose-dependent manner. However, significant increases in uPA mRNA levels were only observed in EVTs treated with the higher concentrations of GnRH I (100 nM) and GnRH II (10 and 100 nM) used in these studies (Fig. 11, A and B).

In agreement with the results obtained using QC-PCR, GnRH I and GnRH II increased uPA protein expression levels in EVT cultures in a dose-dependent manner (Fig. 11, C and D).

3.4.3 GnRH I and GnRH II decrease PAI-1 mRNA and protein expression levels in cultured EVTs

GnRH I and GnRH II decreased PAI-1 mRNA levels in a dose-dependent manner (Fig. 12, A and B).

GnRH I and GnRH II were also found to be capable of reducing PAI-1 protein expression levels in primary cultures of EVTs in a dose-dependent manner (Fig. 12, C and D).

3.4.4 Effects of Cetrorelix on the GnRH I- or GnRH II-mediated regulation of EVT uPA and PAI-1 mRNA and protein expression levels

Cetrorelix decreased the stimulatory effects of GnRH I on uPA mRNA and protein levels in primary cultures of EVTs in a dose-dependent manner (Fig. 13, A and C). In contrast, the ability of GnRH II to increase uPA mRNA and protein expression was not significantly inhibited by
any of the concentrations of Cetrorelix used in these studies (Fig. 13, B and D). Similarly, the inhibitory effects of GnRH I on PAI-1 mRNA and protein levels in EVT cultures were significantly attenuated in cells cultured in the presence of Cetrorelix (100 nM) (Fig. 14, A and C), whereas the addition of this GnRH antagonist to the culture medium had no effect on the down-regulation of PAI-1 mRNA and protein levels mediated by GnRH II (Fig. 14+, B and D).

3.4.5 The ratio of uPA: PAI-1 in conditioned medium of cultured EVT treated with GnRH I or GnRH II

The ratio of uPA/PAI-1 protein levels in conditioned medium of cultured EVT in the presence of increasing concentrations of GnRH I or GnRH II was calculated. The ratio of uPA/PAI-1 in conditioned medium obtained from EVTs cultured in the presence of GnRH II was significantly greater than that obtained from cells treated with GnRH I at all of the concentrations tested (Fig. 15).

3.5. Discussion

In the present studies, we have determined that GnRH I and GnRH II increased uPA-1 and concomitantly decreased PAI-1 mRNA and protein expression levels in primary cultures of EVTs, propagated from explants of first trimester chorionic villi, in a dose- and time-dependent
manner. In addition, Cetrorelix was capable of inhibiting the effects of GnRH I but not GnRH II on uPA and PAI-1 in these cells.

GnRH I and GnRH II have been shown to elicit many diverse biological actions in extrapituitary tissues and cells. For example, GnRH modulates basal and gonadotropin-stimulated steroidogenesis (Peng et al., 1996, Vaananen et al., 1997) and induces transcription of several genes involved in follicular maturation and ovulation in the ovary (Ny et al., 1987, Wong et al., 1992). Furthermore, GnRH I and its synthetic analogs have been shown to inhibit cellular proliferation and induce apoptosis in carcinomas of the ovary (Harris et al., 1991, Emons et al., 1993). Earlier studies demonstrated that breast, ovarian, and endometrial cancers express receptors for GnRH (Friess et al., 1991, Baumann et al., 1993, Dondi et al., 1994, Schally 1994). Data available today suggest that about 50% of breast cancers and approximately 80% of ovarian and endometrial cancers express high-affinity binding sites for GnRH (Fekete et al., 1989). GnRH II was found to suppress tumor cell growth in vitro (Choi et al., 2001). In addition, an autocrine/paracrine function of GnRH has been suggested to exist in the placenta (Merz et al., 1991, Bramley et al., 1992, 1994, Lin et al., 1995), granulosa cells (Minaretzis et al., 1995, Fraser et al., 1996), myometrium (Chegini et al., 1996), and lymphoid cells (Standaert et al., 1992, Ho et al., 1995, Chen et al., 1999). For example, GnRH may act as a luteolytic agent in the
ovary during the regression of the corpus luteum (Goto et al., 1999). Furthermore, in addition to
the ability of GnRH I and GnRH II to regulate human chorionic gonadotropin production in the
human placenta (Siler-Khodr et al., 2001), our studies indicate that these two hormones may also
modulate the invasive capacity of human trophoblast in vitro.

Plasminogen activators and their inhibitors (PAIs) have been identified in placenta are
considered to be key participants in the balance of proteolytic and antiproteolytic activities that
regulate extracellular matrix turnover. They are thought to be involved in various processes
known to be associated with extensive tissue remodeling and cellular migration (Sappino et al.,
1993). Direct focal degradation of proteins involved in cell-cell and cell-matrix interactions by
plasmin has been described (Saksela et al., 1988). Complex control of the plasminogen activator
cascade has been shown to be required for the movement and reorganization of cells and matrix
in events such as trophoblast invasion.

To date, the mechanism(s) by which GnRH regulates uPA/PAI expression in villous
cytotrophoblasts has not been determined. One possible mechanism is through the GnRH
I-mediated increase in the transcription factor, AP-1 (Wurmbach et al., 2001). Multiple AP-1
binding sites have been detected in the promoter regions of both the PAI and uPA human genes
(Irigoyen et al., 1999). GnRH I has also been shown to stimulate cAMP production in mixed
pituatory cell cultures, suggesting a potential relationship between GnRH and this intracellular secondary signaling pathway (Borgeat et al., 1972, Bourne 1988). An increase in intracellular cAMP concentration decreases the levels of PAI-1 expression in various cell lines (Riccio et al., 1988, Slivka et al., 1991). The intracellular signaling events mediated by GnRH II remain to be elucidated.

The human placenta contains specific binding sites for GnRH I (Currie et al., 1981). Recently, mRNA transcripts encoding the GnRH I receptor have been detected in human placental tissues and trophoblastic cell cultures (Lin et al., 1995, Wolfahnt et al., 1998, Nathwani et al., 2000). Although the full-length mRNA transcript encoding the full-length human GnRH II receptor has not been characterized, the presence of GnRH II receptor immunoreactivity in the human pituitary and brain has been demonstrated (Neil et al., 2001, Millar et al., 2001). Furthermore, GnRH II receptor mRNA transcripts have been detected in the human term placenta (Neil et al., 2001). Our results show that Cetrorelix, an antagonist specific for the GnRH I receptor (Millar et al., 2001, Neil et al., 2002), is able to significantly block the effects of GnRH I on uPA mRNA and protein levels. In contrast, the stimulatory effects of GnRH II on levels of uPA protein were not significantly reduced by this GnRH antagonist. These observations suggest that these effects
are mediated by distinct receptors and that minimal cross-reactions occur between GnRH I and
-II and their specific receptors in human trophoblasts.

The biological effects of GnRH II have been shown to be greater than those observed with
GnRH I in extrapituitary cells. For example, GnRH II had a much greater effect on the inhibition
of tumor cell proliferation than GnRH I and its agonists (Grundker et al., 2002). Similarly,
GnRH II was capable of increasing uPA mRNA and protein expression levels at lower doses and
in shorter time interval than GnRH I in our primary cultures of extravillous cytotrophoblasts.
These effects may be mediated by GnRH II binding to a specific high-affinity receptor with
greater potency than GnRH I and/or GnRH II may be degraded at a slower rate compared with
GnRH I (Standaert et al., 1992). Receptor binding assays in COS-7 cells have demonstrated that
GnRH II is highly selective for the type II receptor in nonhuman primates (Millar et al. 2001).

In summary, our findings demonstrated that both GnRH I and GnRH II are capable of
up-regulating uPA and down-regulating PAI-1 in trophoblasts in vitro. Thus, the two types of
GnRH produced in the placenta may facilitate invasion by virtue of increasing the ratio of
uPA/PAI-1 and provide further evidence that members of GnRH family act in an autocrine or
paracrine manner in extrapituitary tissues and cells.
419 bp internal standard cDNA

5'-CTGTGACTGTCTAAATGAGG-3'

622 bp target cDNA

5'-CTGTGACTGTCTAAATGAGG-3'  GACGATGTAGTCCTCCTTCTT-3'

Figure 8. Representative schematic diagram summarizing the construction of a competitive PCR primer for uPA. An internal standard fragment was constructed by deletion of a 203-bp fragment from the specific target cDNA to be detected.
A.

Volume

count(mm²)

622 bp

419 bp

(target) (internal standard)

(pg/ul) 8 4 2 1 0.5 0.25 0.125

B.

Volume

count(mm²)

687 bp

464 bp

(target) (internal standard)

(pg/ul) 8 4 2 1 0.5 0.25 0.125
C.

Log(amounts of target cDNA (pg))

D.

Log(amounts of target cDNA (pg))
Figure 9. Standard curves generated for uPA and PAI. Photomicrograph of an ethidium bromide-stained gel containing uPA or PAI-1 specific PCR products generated by the coamplification of a fixed amount of target cDNA (1 μl) and serial dilutions of concentrations of competitive cDNA (8, 4, 2, 1, 0.5, 0.25, 0.125 pg/μl) (A and B, upper panel). The two lines cross in the range of 0.5–1 pg/μl and 1 pg/μl internal standard cDNA added for uPA and PAI-1, indicating that approximated 1 pg uPA and PAI-1 cNDA could be detected after RT of 1 μg total RNA (A and B, lower panel). Increasing amounts of target cDNA were coamplified with 1 pg/μl competitive cDNA. The intensities of the ratio of target and competitive cDNA generated from these reaction mixtures were determined (C and D, upper panel). The log ratios of target competitive product density plotted against the log amount of target initially added to the PCR reactions are shown in the graphs below (C and D).
A.

\[ \text{mRNA (pg/ul)} \]

\[ \text{GnRH I 100 nM (hr) 0 3 6 12 24 48} \]

B.

\[ \text{mRNA (pg/ul)} \]

\[ \text{GnRH II 100 nM (hr) 0 3 6 12 24 48} \]
Figure 10. QC-PCR analysis of the effects of GnRH I or GnRH II on uPA and PAI mRNA levels in EVTs. Time-dependent effects on uPA (A and B) and PAI-1 (C and D) were determined by culturing the cells in the presence or absence of GnRH I (A and C) or GnRH II (B and D) for 0–48 h (lanes 1–6, respectively). Representative photomicrographs of the corresponding ethidium-stained gels are shown in the upper panels. A 100-bp DNA ladder is shown in lane M with the size of the target and competitive cDNAs indicated on the left. The gels were analyzed using UV densitometry. Data are presented as the means of five individual experiments and are presented as the mean ± SE (a, \( P < 0.001 \) vs. untreated control; b, \( P < 0.05 \) vs. untreated control) in the bar graphs shown in the lower panels.
Figure 11. Effects of GnRH I and GnRH II on uPA mRNA and protein expression levels in cultured EVTs. Dose-dependent effects were determined by culturing the cells in increasing concentrations (0–100 nM; lanes 1–5, respectively) of GnRH I (A) or GnRH II (B) for 24 h. Representative photomicrographs of the corresponding ethidium-stained gels are shown in the upper panels. A 100-bp DNA ladder is shown in lane M with the size of the expected PCR products indicated on the left. ELISA analysis of uPA expression level in conditioned medium of isolated EVTs cultured in the presence of an increasing concentration of GnRH I (C) or GnRH II (D). One milligram of protein from conditioned medium was used in each ELISA. Data are shown as the means of five individual experiments and are presented as the mean ± SE.
\( P < 0.001 \) vs. untreated control) in the bar graphs shown in the lower panels.

A.

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**Figure 12.** Effects of GnRH I and GnRH II of PAI-1 mRNA and protein expression levels in cultured EVTs. QC-PCR analysis of PAI-1 mRNA levels were determined by culturing the cells in increasing concentrations (0–100 nM; lanes 1–5, respectively) of GnRH I (A) or GnRH II (B) for 24 h. Representative photomicrographs of the corresponding ethidium-stained gels are shown in the *upper panels*. A 100-bp DNA ladder is shown in lane M with the size of the expected PCR products indicated on the *left*. The blots were analyzed using UV densitometry. ELISA analysis of PAI-1 expression level in conditioned medium of isolated EVTs cultured in the presence of an increasing concentration of GnRH I (C) or GnRH II (D). One milligram of protein from conditioned medium was used in each ELISA. Data are shown as the means of five individual experiments and are presented as the mean ± SE (a, \( P < 0.001 \) vs. untreated control. b, \( P < 0.05 \) vs. untreated control) in the bar graphs shown in the *lower panels*. 
C.

GnRH I  0  100nM  100nM  100nM  100nM
Cetrorelix  0  0  1nM  10nM  100nM

D.

GnRH II  0  100nM  100nM  100nM  100nM
Cetrorelix  0  0  1nM  10nM  100nM
Figure 13. Effects of Cetrorelix on the GnRH I- or GnRH II-mediated regulation of EVT uPA mRNA and protein expression levels. QC-PCR analysis of the EVTs. Cells were cultured in the presence of 100 nM GnRH I (A) or GnRH II (B) and an increasing amount of Cetrorelix. Representative photomicrographs of the corresponding ethidium stained gels are shown in the upper panels. A 100-bp DNA ladder is shown in lane M with the size of the target and competitive cDNAs indicated on the left hand side. The blots were analyzed using UV densitometry. ELISA analysis of uPA expression level in conditioned medium of isolated EVT cultured in the presence of an increasing concentration of GnRH I (C) or GnRH II (D). One milligram of protein from the conditioned medium was loaded in each ELISA reaction. Data are shown as the means of five individual experiments, and are presented as the mean ± SE (a, $P < 0.001$ vs. 100 nM GnRH, b, $P < 0.05$ vs. 100 nM GnRH) in the bar graphs shown in the lower panels.
Figure 14. Effects of Cetrorelix on the GnRH I- or GnRH II-mediated regulation of EVT PAI-1 mRNA and protein expression levels. QC-PCR analysis of EVTs. Cells were cultured in the presence of 100 nM GnRH I (A) or GnRH II (B) and an increasing amount of Cetrorelix. Representative photomicrographs of the corresponding ethidium stained gels are shown in the upper panels. A 100-bp DNA ladder is shown in lane M with the size of the target and competitive cDNAs indicated on the left. The blots were analyzed using UV densitometry. ELISA analysis of PAI-1 expression level in conditioned medium of isolated EVT cultured in the presence of GnRH I (C) or GnRH II (D) and an increasing amount of Cetrorelix. One milligram of protein from the conditioned medium was loaded in each ELISA reaction. Data are shown as the means of five individual experiments, and are presented as the mean ± SE (a, $P < 0.001$ vs. 100 nM GnRH, b, $P < 0.05$ vs. 100 nM GnRH) in the bar graphs shown in the lower panels.
**Figure 15.** Line graph depicting the ratio of uPA/PAI-1 expression levels in conditioned medium of EVTs cultured in the presence of increasing concentrations of GnRH I or GnRH II (0–100 nM).
4.1 ABSTRACT

To date, the factors capable of regulating the coordinate expression of the urokinase-type plasminogen activator (uPA) and its endogenous inhibitor, plasminogen activator inhibitor (PAI-1) at the maternal-fetal interface remain poorly characterized. In these studies, we have examined the ability of the classical Gonadotropin-Releasing Hormone (GnRH I) and the second mammalian form of GnRH (GnRH II) to regulate uPA and PAI-1 mRNA and protein expression levels in primary cultures of stromal cells isolated from first trimester decidual tissues using quantitative competitive-PCR and ELISA, respectively. GnRH I and GnRH II increased uPA mRNA and protein expression levels in these primary cell cultures in a dose- and time-dependent manner. In contrast, GnRH I increased whereas GnRH II decreased PAI-1 mRNA and protein expression levels in these primary cell cultures. Cetrorelix, a GnRH receptor antagonist, inhibited the regulatory effects of GnRH I, but not GnRH II on uPA and PAI-1 expression levels in these cells. Taken together, these observations suggest that GnRH I and GnRH II differentially regulate the balance between uPA and PAI-1 expression levels in the human decidua possibly via the activation of distinct intracellular signaling pathways.
4.2 INTRODUCTION

The establishment of a successful pregnancy is dependent upon the coordinated development of the implanting embryo and the maternal endometrium (Tabibzadeh et al., 1991, Paria et al., 2002). In particular, the blastocyst must have attained the ability to attach to endometrial epithelium, and subsequently invade into the underlying decidua. However, unlike tumor cells, trophoblast invasion into the underlying maternal tissues is highly regulated and errors can have severe consequences for the health of both the mother and fetus (Graham et al., 1992, Paradinas et al., 1995). The terminal differentiation of the endometrial stroma into decidua, which begins in the secretory phase of the menstrual cycle and continues into early pregnancy, is believed to be a critical event in the development of a uterine environment that is capable of fulfilling this embryoregulatory role (Graham et al., 1992, Lockwood et al., 1999).

The urokinase plasminogen activator (uPA) and its endogenous inhibitor, plasminogen activator inhibitor (PAI)-1, play key roles in the highly regulated series of remodeling events that occur in the endometrium in preparation for pregnancy (Feinberg et al., 1989, Floridon et al., 1999, Schatz et al., 1999). In particular, uPA is believed to mediate, at least in part, the degradation of the endometrial extracellular matrix (ECM) underlying the development of the decidua and the regulated invasion of extravillous cytotrophoblasts. The proteolytic activity of uPA at the maternal-fetal interface is counter-balanced, in both an autocrine and paracrine
manner, by PAI-1 secreted by the decidual cells and the subpopulation of highly invasive extravillous cytotrophoblasts. Although it is recognized that the remodeling of the decidual ECM is a critical event in the establishment of pregnancy, the factors capable of regulating the coordinate expression of uPA and PAI-1 in this dynamic tissue remain poorly characterised.

We have recently determined that both the classical form of mammalian GnRH (GnRH I) and the second mammalian form of this hormone (GnRH II) are key regulators of uPA-1 and PAI-1 mRNA and protein expression levels in extravillous cytotrophoblasts propagated from first trimester placental explants (Chou et al., 2002). In particular, GnRH I and GnRH II increased uPA and concomitantly decreased PAI-1 expression levels in these primary cell cultures. As GnRH I and GnRH II are secreted by both the human placenta and endometrium (Raga et al., 1998, Cheon et al., 2001, Siler-Khodr et al., 2001, Kikkawa et al., 2002), it is tempting to speculate that these two hormones may be key regulators of the proteolytic degradation of ECM that occurs at the maternal-fetal interface during early pregnancy. To gain a better understanding of the role(s) of GnRH I and GnRH II in this developmental process, we have examined the ability of GnRH I and GnRH II to regulate uPA and PAI-1 mRNA and protein expression levels in primary cultures of stromal cells isolated from first trimester decidual tissues in a dose-and time-dependent manner.
4.3 MATERIALS AND METHODS

4.3.1 Tissues

Tissue samples of first trimester *decidua parietalis* were obtained from women undergoing elective termination of pregnancy. The use of these tissues was approved by the committee for ethical review of research involving human subjects, University of British Columbia. All patients provided informed written consent.

4.3.2 Cell Isolation and Culture

Stromal cells were isolated from the decidual tissue samples by enzymatic digestion and mechanical dissociation using a protocol modified from that reported by Shiokawa et al (1996). Briefly, the decidual tissue samples were minced and subjected to 0.1% collagenase (type IV, sigma Chemical Co, St Lois, MO) and 0.1% hyaluronidase (type I-S) digestion in a shaking water bath at 37 °C for 60 min. The cell digest was then passed through a nylon sieve (38 μm). The isolated glands and any undigested tissue fragments were retained on the sieve, and the eluate containing the stromal cells was collected in a 50ml tube. The stromal cells were then pelleted by centrifugation at 800 × g for 10 min at room temperature. The cell pellet was washed once with DMEM containing 10% fetal bovine serum (FBS) before being resuspended and plated in DMEM containing 25mM glucose, L-glutamine, antibiotics (100U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% FBS, 17β-estradiol (10 nM; Sigma
Chemical Co) and progesterone (1 μM, Sigma Chemical Co). The culture medium was replaced 30 min after plating to reduce epithelial cell contamination. The purity of the decidual stromal cell cultures was determined by immunocytochemical staining for vimentin, cytokeratin, muscle actin and factor VIII (data not shown). These cellular markers have been used to determine the purity of human endometrial cell cultures (Irwin JC et al. 1989). As defined by these criteria, the decidual stromal cell cultures used in these studies contained < 1% epithelial or vascular cells.

4.3.3 Hormone Treatments

Decidual stromal cells (passage 2) were plated in 35 mm² tissue culture dishes (Becton Dickinson and Co, Franklin Lakes, NJ) at a density of 1 x 10⁶ cells/dish and grown to 80% confluency. The cells were then cultured in the presence of increasing concentrations of GnRH I or GnRH II (0, 0.1, 1, 10, or 100nM) for 24 h or a fixed concentration of GnRH I or GnRH II (100 nM) for 0, 3, 6, 12, 24 or 48 h. In addition, decidual stromal cell cultures were treated with a combination of GnRH I or GnRH II (100 nM) and increasing concentrations (0, 1, 10, or 100 nM) of the GnRH I antagonist, Cetrorelix, for 24h. Cells treated with vehicle (0.1% ethanol) alone served as a control for these experiments.

The viability of the decidual cell cultures was determined by a trypan blue exclusion assay.
4.3.4 Primer Design

The design and nucleotide sequences of the primer sets specific for uPA, PAI-1 used in the QC-PCR analysis or the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were used to quantify and assess the integrity of the total RNA samples, have been described in detail elsewhere (Chou et al., 2002).

4.3.5 RNA Preparation and RT-PCR

Total RNA was prepared from the decidual cell cultures using a RNeasy Mini Kit (Qiagen, Inc, Mississauga, Canada) using a protocol recommended by the manufacturer. The concentration of total RNA present in each of the extracts was quantified by optical densitometry (260/280nm) using a Du-64 UV-spectrophotometer (Beckman Coulter). An aliquot (1μg) of the total RNA extracts prepared from the decidual stromal cells was reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Oakville, Canada).

PCR was performed using template cDNA generated from the total RNA extracts prepared from cultures of untreated decidual stromal cells and the primers sets specific for GAPDH, uPA or PAI-1. The PCR conditions were as follows: 1 min at 94 C, 1 min at 57.5 C or 56 C for PAI-1 and uPA, respectively; and 1.5 mins at 72 C followed by a final extension at 72 C for 15 min.
The cycles were repeated 20-35 times. A linear relationship between the yield of the PCR products and number of cycles performed was observed after 27 cycles for uPA and 30 cycles for PAI-1 (data not shown).

The resultant PCR products for uPA and PAI-1 were separated using gel electrophoresis and visualised by ethidium bromide staining (data not shown). An aliquot of these uPA and PAI-1 PCR products were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequence analysis to confirm the specificity of the primers. These clones were also used to generate target or internal standard uPA and PAI-1 cDNA fragments by standard molecular biology techniques.

4.3.6 Quantitative Competitive-Polymerase Chain Reaction) QC-PCR

The QC-PCR strategy employed in these studies is based upon the competitive co-amplification of a known amount of an internal standard specific for uPA or PAI-1 added to aliquots of the first strand cDNA prepared from our primary cultures of decidual stromal cells (Chou et al., 2002, Jin et al., 1994, Chou et al., 2003).

To determine the optimal amounts of the internal standards to be used in the QC-PCR analysis, PCR reaction mixtures containing a fixed amounts of the target uPAI-1 or PAI-1 cDNAs (1μl) and decreasing concentrations of the corresponding internal standard cDNAs (8-0.0625 pg/μl for uPA or 8-0.125 pg/μl PAI-1, respectively) were prepared. PCR was then
performed using these cDNA mixtures and the distinct uPA or PAI-1 primer sets under the optimized conditions described above.

An aliquot (10 μl) of the resultant uPA and PAI-1 PCR products were separated by electrophoresis in a 1% agarose gel and visualised by ethidium bromide staining (Fig. 16). The intensity of the ethidium bromide staining of the PCR products was analysed using UV densitometry (Biometra, Whiteman Co., Gottigen, German). Volume counts (mm$^2$) of the PCR products were then determined using the Scion Image computer software (Scion Image Co, Frederick, MD). The absorbance values obtained for each of the target and corresponding internal standard were plotted against the amount of internal standard added to the initial reaction mixtures, with the point of interception on these line graphs being taken as the optimal amount of internal standard to used in the QC-PCR analysis (Fig 16). Based upon these observations, uPA or PAI-1 internal standard cDNAs were added to aliquots of the first strand cDNA generated from the human decidual cells, to be used in the QC-PCR analysis, at concentrations of 1 pg/μl or 0.5 pg/μl, respectively.

PCR was also used to co-amplify the optimized amounts of uPA and PAI-1 internal standard cDNAs and increasing amounts of the corresponding target cDNAs (0.0625-8 pg/μl). The ratios of the intensity of the ethidium bromide staining of the resultant target: internal standard PCR products generated in each tube were logarithmically transformed and plotted against the log
amount of target cDNA initially added to the PCR reaction. Representative standard curves
generated for uPA and PAI-1 are presented in Fig. 16. These standard curves were highly
reproducible and linear. The values obtained from these regression curves \( y = b + mx \) were
subsequently used to quantify the levels of uPA and PAI-1 mRNA transcripts present in our
decidual stromal cell cultures.

QC-PCR was performed using the uPA or PAI-1 primer sets and 1 \( \mu l \) of the first strand cDNA
synthesized from each of the treated decidual stromal cell cultures containing the optimized
amount of the corresponding internal standard cDNA under the PCR conditions described above.
The ratios of the intensity of ethidium bromide staining of the resultant target: internal standard
PCR products were logarithmically transformed and compared with the values obtained from
the standard curves.

4.3.7 ELISA

The expression levels of uPA and PAI-1 in the conditioned medium collected from the decidual
stromal cell cultures were measured using ELISA kits for uPA or PAI-1 (American Diagnostica,
Inc. Greenwich, CA). uPA was detected in the conditioned culture medium with a mean
intra-assay and inter-assay coefficient of variation of 4.9% and 8.2%, respectively whereas
PAI-1 was detected in the conditioned medium with a mean intraassay and interassay coefficient
of variation of 6.1% and 8.8%, respectively. All samples were assayed in duplicate.
4.3.8 Statistical Analysis

The absorbance values obtained from the ethidium bromide stained gels were subjected to statistical analysis using GraphPad Prism 2 computer software (San Diego, CA, USA). 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 ± the standard error of the mean (SEM) obtained from 5 independent experiments.
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Volume count (m^2)

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Volume count (m^2)

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C.

Log amounts of target cDNA (pg)

D.

Log amounts of target cDNA (pg)
Figure 16. Preparation of standard curves for the QC-PCR analysis of uPA and PAI-1 mRNA levels in decidual stromal cells. Panels A and B: Photomicrographs of ethidium bromide stained gels containing PCR products generated using a fixed amount of target cDNA and increasing amounts of the corresponding internal standard. The sizes of the resultant PCR products relative to a 100bp ladder (MW) are indicated. The intensity of the ethidium bromide staining of these PCR products was determined by UV densitometry and the resultant absorbance values of the ethidium plotted against the amount of internal standard added to each PCR reactions in the line graphs below.

Panels C and D. Photomicrographs of ethidium bromide gels containing PCR products generated using a fixed amount of internal standard and decreasing amounts of target uPA or PAI-1 cDNAs. The intensity of the ethidium bromide staining of the target and internal standard PCR products was determined by UV densitometry. The linear relationship between the logarithmically transformed ratios of target:internal standard cDNAs and the amount of target cDNA added to the initial PCR are shown in the graphs below.
4.4 RESULTS

4.4.1 GnRH I and GnRH II regulate uPA mRNA and protein expression levels in primary cultures of decidual stromal cells

u-PA mRNA transcripts and protein expression were detected in all of the decidual stromal cell cultures. The addition of vehicle to the culture medium had no significant effect on the uPA mRNA and protein expression levels in these primary cell cultures at any of the time points examined in these studies (data not shown). In contrast, GnRH I and GnRH II increased uPA mRNA levels and protein expression in the cultured decidual stromal cells in a dose-dependent manner (Fig. 17).
C.

uPA
(unit/mg protein) + SEM

GnRH I 0 0.1nM 1nM 10nM 100nM

D.

uPA
(unit/mg protein) + SEM

GnRH II 0 0.1nM 1nM 10nM 100nM

121
Figure 17. uPA mRNA and protein expression levels in decidual cells cultured in the presence of increasing concentrations of GnRH I or GnRH II. Panels A and B: Representative photomicrographs of ethidium bromide stained gels containing QC-PCR products generated using template cDNA synthesised from decidual cells cultured in the presence of 0, 0.1, 1, 10 or 100 nM for 24h (lanes 1-5, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked on the left hand-side of the photomicrograph. The intensity of the ethidium bromide staining of the PCR products was determined by UV densitometry and the resultant absorbance values used to calculate the ratio of target to internal standard cDNA for each QC-PCR reaction. The results derived from this analysis as well as from four other independent studies (data not shown) are represented (mean ± SEM n=5) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control).

Panels C and D: ELISA analysis of uPA expression level in conditioned medium of these decidual stromal cells. One milligram of protein from conditioned medium was used in each ELISA. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs. untreated control) in the bar graphs.
A significant increase in uPA mRNA levels was detected in decidual stromal cells cultured in the presence of GnRH I for 12 h with maximum levels being observed after 24 h of culture under these experimental conditions (Fig. 18). Although there was a slight decline in uPA mRNA levels in decidual cells cultured in the presence of GnRH I for 48 h, the levels of this mRNA transcript were still significantly greater than those detected in the 0 h control. A significant and progressive increase in uPA protein expression levels was also detected in these cell cultures until the termination of these studies at 48 h.

The levels of the uPA mRNA transcript in the decidual stromal cells cultured in the presence of GnRH II were significantly higher after 12 h of culture and continued to increase until the termination of these studies at 48 h (Fig. 18). A coordinate increase in the expression levels of uPA in these decidual stromal cells was also observed at all of the time points examined (Fig. 18).
Figure 18: Time-dependent effects of GnRH I or GnRH II on uPA mRNA and protein expression levels in decidual cells. Panels A and B: QC-PCR analysis of uPA mRNA levels in decidual cells cultured in the presence of GnRH I or GnRH II (100 nM) for 0, 3, 6, 12, 24 or 48 h (lanes 1-6, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control).

Panels C and D: ELISA analysis of uPA expression level in conditioned medium of these
decidual stromal cells. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs untreated control) in the bar graphs.

4.4.2 Differential effects of GnRH I and GnRH II on PAI-1 mRNA and protein expression levels in decidual stromal cells.

PAI-1 mRNA transcripts and protein expression were detected in all of the decidual stromal cell cultures. The addition of vehicle to the culture medium had no significant effect on PAI-1 mRNA or protein expression levels in these cells at any of the time points examined in these studies (data not shown).

GnRH I increased, whereas GnRH II decreased PAI-1 mRNA and protein expression levels in our primary cultures of decidual stromal cells in a dose-dependent manner (Fig. 19).

There was a significant increase in PAI-1 mRNA and protein expression levels in decidual stromal cells cultured in the presence of GnRH I for 3 h with maximum levels being observed in cells after 12 h of treatment (Fig. 20). There was a subsequent and progressive decline in PAI-1 mRNA and protein expression levels in these primary cell cultures until the termination of these studies at 48 h.

In contrast, the addition of GnRH II to the culture medium of the decidual stromal cells resulted in a decrease in PAI-1 mRNA and protein expression levels within 3 h of treatment. PAI-1 mRNA and protein expression levels in these cells continued to decline until 24 h after which, there was an increase in both PAI-1 mRNA and protein levels (Fig. 20).
Figure 19: PAI-1 mRNA and protein expression levels in decidual cells cultured in the presence of increasing concentrations of GnRH I or GnRH II. Panels A and B: QC-PCR analysis of PAI-1 mRNA levels in decidual cells cultured in the presence of 0, 0.1, 1, 10 or 100 nM for 24h (lanes 1-5, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control)
Panels C and D: ELISA analysis of PAI-1 expression level in conditioned medium of these decidual stromal cells. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs untreated control) in the bar graphs.
A.

![Graph A]

B.

![Graph B]
Figure 20: Time-dependent effects of GnRH I or GnRH II on PAI-1 mRNA and protein expression levels in decidual cells. Panels A and B: QC-PCR analysis of PAI-1 mRNA levels in decidual cells cultured in the presence of GnRH I or GnRH II (100 nM) for 0, 3, 6, 12, 24 or 48 h (lanes 1-6, respectively) and the primer sets specific for PAI-1. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs untreated control)
Panels C and D: ELISA analysis of PAI-1 expression level in conditioned medium of these decidual stromal cells. Data are shown as the means of five independent assays± SEM (a, P<0.001; b, P<0.05 vs untreated control) in the bar graphs.
4.4.3. Effects of Cetrorelix on the GnRH I- or GnRH II-mediated regulation of uPA and PAI-1 mRNA and protein expression levels in decidual stromal cells

Cetrorelix inhibited the stimulatory effects of GnRH I on uPA mRNA and protein expression levels in cultured decidual stromal cells in a dose-dependent manner. In contrast, Cetrorelix had no significant effect on the GnRH II-mediated increase in uPA mRNA and protein levels in these primary cell cultures at any of the concentrations examined in these studies (Fig. 21).

Cetrorelix also inhibited GnRH I-mediated decrease PAI-1 mRNA and protein expression levels in our decidual stromal cell cultures in a dose-dependent manner. In contrast, the addition of this GnRH I antagonist to the culture medium had no significant effect on the uPA mRNA and protein levels in decidual stromal cells cultured in the presence of GnRH II, at any of the concentrations examined in the studies (Fig. 22).
A.

B.
Figure 21: Effects of Cetrorelix on uPA mRNA and protein expression levels in decidual stromal cells cultured in the presence of GnRH I or GnRH II. Panels A and B: QC-PCR analysis of uPA mRNA levels in untreated decidual cells (lane 1) or cells cultured in the presence of a fixed amount of GnRH I or GnRH II (100 nM) and an increasing amount (0, 1, 10 or 100 nM) of the GnRH antagonist, Cetrorelix, (lanes 2-5, respectively) for 24h. The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. treatment with GnRH I or GnRH II alone).

Panels C and D: ELISA analysis of uPA expression level in conditioned medium of these decidual stromal cells. Data are shown as the means of five independent assays ± SEM. (a, P<0.001 vs treatment with GnRH I or GnRH II alone) in the bar graphs.
C.

![Bar graph showing PAI-1 levels with SEM](image)

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D.

![Bar graph showing PAI-1 levels with SEM](image)

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**Figure 22:** Effects of Cetrorelix on PAI-1 mRNA and protein expression levels in decidual stromal cells cultured in the presence of GnRH I or GnRH II. Panels A and B: QC-PCR analysis of PAI-1 mRNA levels in untreated decidual cells (lane 1) or cells cultured in the presence of a fixed amount of GnRH I or GnRH II (100 nM) and an increasing amount (0, 1, 10 or 100 nM) of the GnRH antagonist, Cetrorelix, (lanes 2-5, respectively) for 24h. The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. treatment with GnRH I or GnRH II alone)

Panels C and D: ELISA analysis of PAI-1 expression level in conditioned medium of these decidual stromal cells. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs treatment with GnRH I or GnRH II alone) in the bar graphs.
4.4.4 The ratio uPA: PAI-1 expression levels in conditioned medium of decidual stromal cells treated with GnRH I or GnRH II

The ratio of uPA/PAI-1 expression levels in the samples of conditioned media obtained from decidual cells cultured in the presence of GnRH II was significantly greater than those obtained from cells treated with GnRH I at all of the hormone concentrations examined in these studies (Fig. 23).
Figure 23: Line graph depicting the ratio of uPA/PAI-1 protein expression levels in samples of conditioned medium obtained from decidual stromal cells cultured in the presence of increasing concentrations of GnRH I or GnRH II (0-100nM) for 24 h.
4.5 DISCUSSION

Here we report that GnRH I and GnRH II are both capable of increasing uPA expression in human decidual stomal cells. In contrast, GnRH I increased whereas GnRH II caused a significant decrease in PAI-1 mRNA and protein expression levels in these primary cell cultures. We have recently determined that both GnRH I and GnRH II increase uPA-1 and concomitantly decrease PAI-1 mRNA and protein expression levels in primary cultures of human extravillous cytотrophoblasts (Chou et al., 2002). In addition, we have found that GnRH I increases the expression levels of matrix metalloproteinase-2 and -9 expression levels in stromal cells isolated from first trimester decidual tissues (Chou et al., 2003), two other proteases operative at the maternal fetal interface during pregnancy (Fata et al., 2000. Vu et al., 2000). GnRH I has also been shown to be capable of decreasing the levels of the tissue specific inhibitor of matrix metalloproteinases (TIMP)-1 and TIMP-3 present in human endometrial stromal cells allowed to undergo steroid-mediated decidualisation in vitro (Raga et al., 1999). In view of these observations, it is tempting to speculate that GnRH I and GnRH II play key regulatory roles in the proteolytic degradation of the ECM of the endometrial stroma, a prerequisite for decidualisation and the subsequent invasion of trophoblasts (Graham et al., 1992, Paradinas et al., 1995).

GnRH II has been shown to mimic the biological actions of GnRH I (Neill et al., 2002). Often,
the effects of GnRH II on extrapituitary tissues and cells appear to be significantly greater than those observed with native GnRH I and/or its synthetic analogues. For example, the antiproliferative effects of GnRH II on human endometrial and ovarian cancer cells were significantly greater than those observed in cells cultured in the presence of equivalent concentrations of GnRH I or the GnRH I agonist, Triptorelin (Grundker et al., 2002). GnRH II has also been shown to have a more potent inhibitory effect on the secretion of hCG by human term placenta and the steroidogenic capacity of granulosa-lutein cells in vitro (Siler-Khodr et al., 2001, Kang et al., 2001). However, we have determined that GnRH I and GnRH II have differential effects on PAI-1 mRNA and protein expression levels in primary cultures of decidual stromal cells. To our knowledge, these studies are the first to demonstrate that GnRH I and GnRH II are capable of having differential biological actions on mammalian cells and suggest that the regulatory effects of these two hormones may be tissue/cell-specific. Although the molecular mechanisms underlying the differential effects of GnRH I and GnRH II on PAI-1 mRNA and protein expression levels in these cell cultures have yet to be elucidated, our observations suggest that these distinct biological actions may be owing to differences in the binding affinity of GnRH I and GnRH II to one or more GnRH receptors and/or the activation of distinct intracellular signaling pathways.

Although significant GnRH I receptor mRNA levels have not been detected in total RNA
extracts prepared from human endometrial tissues obtained at any stage of the menstrual cycle or in early pregnancy (Ikeda et al., 1997), recent studies have demonstrated that this mRNA transcript is present in primary cultures of human endometrial stromal cells (Huang et al., 2003). In addition, low binding affinity/high capacity binding and high binding affinity/low capacity binding sites for GnRH I have been detected in normal and malignant human endometrial cells suggesting that two distinct GnRH receptors are present in this dynamic tissue (Takeuchi et al., 1998). Recently, a gene encoding a second receptor for GnRH (GnRHRII) has been identified in the human genome (Neill 2002, Millar et al., 2001). Although a full-length mRNA transcript encoding this second form of human GnRHR has not been isolated, GnRHRII mRNA transcripts have been detected in the human endometrium and placenta (Grundker et al., 2002).

The ability of Cetrorelix, an antagonist believed to be specific for the GnRH I receptor, to inhibit the regulatory effects of GnRH I, but not GnRH II, on uPA and PAI mRNA levels in stromal cells isolated from first trimester decidual tissues, provides further evidence that the biological actions of these two hormones may be elicited by distinct receptors.

Prolonged exposure to GnRH I resulted in a decrease in uPA and PAI-1 mRNA and protein expression levels in the primary cultures of decidual stromal cells. Similarly, the inhibitory effects of GnRH II on PAI-1 mRNA and protein expression levels in decidual stromal cells were reduced with time in culture. This biphasic effect may be attributed to the desensitization of
GnRHR-I, a biological phenomenon often observed in the GnRH I-stimulated pituitary cells (McArdle et al., 2002). GnRH I binding to its receptor activates several intracellular signaling pathways including the protein kinase A, protein kinase C and/or mitogen activated protein kinases (ERK 1/2 MAP kinases) cascades (Cheng et al., 2000). Recent studies indicate that the activation of ERK 1/2 signalling cascade by GnRH I involves the transactivation of the epidermal growth factor receptor (EGFR) (Shah et al., 2003). Interestingly, there is a marked increase in EGFR expression in the endometrial stroma as it undergoes decidualisation, with maximum levels being detected in decidua cells present at the maternal-fetal interface (Lockwood et al., 2000). EGFR agonists have also been shown to increase PAI-1 expression levels in human endometrial stromal cells undergoing steroid-mediated decidualisation in vitro (Lockwood et al., 2000, 2001). In contrast, invasive extravillous cytotrophoblasts do not express significant levels of EGFR (Johki et al., 1994, Aboagye-Mathiesen et al., 1997). Thus, the differential effects of GnRH on PAI-1 mRNA and protein expression levels in primary cultures of decidual stromal cells and extravillous cytotrophoblasts may be owing to the presence or absence of EGR in these two cell types, respectively.

In summary, we have determined that GnRH I and GnRH II increase uPA mRNA and protein expression levels in primary cultures of stromal cells isolated from first trimester decidual tissues in a dose-, and time-dependent manner. In contrast, GnRH I increased, whereas GnRH
II decreased, the expression levels of the endogenous inhibitor of uPA, PAI-1 in these cell cultures. These findings strengthen our hypothesis that the two forms of GnRH secreted by the placenta and endometrium play key regulatory roles in the ECM remodeling events that occur at the maternal-fetal interface during pregnancy in the human.
PART V. Effects of GnRH on MMP-2, and MMP-9 and TIMP in Human Decidual Stromal Cells

5.1 Abstract

Matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue-specific inhibitor of matrix metalloproteinases (TIMPs), play key roles in the cyclic remodeling events that occur in the human endometrium in preparation for pregnancy. To date, the factors capable of regulating the expression of MMPs and TIMPs in the human decidua remain poorly characterized. The spatiotemporal expression of GnRH in the human endometrium during the menstrual cycle and early pregnancy suggests that this hormone may have a regulatory role in the development of this dynamic tissue. In view of these observations, we have examined the ability of GnRH to regulate MMP-2, MMP-9, and TIMP-1 mRNA levels in primary cultures of human decidual stromal cells using a quantitative competitive PCR strategy. GnRH was capable of increasing MMP-2 and MMP-9 mRNA levels in these primary cell cultures in a dose-dependent manner. The GnRH antagonist, antide, was capable of inhibiting the GnRH-mediated increase in the levels of the MMP-2 and MMP-9 mRNA transcripts present in these decidual stromal cells in a dose-dependent manner. In contrast, GnRH or antide did not have a significant effect on TIMP-1 mRNA level in these primary cell cultures at any of the concentrations used in these studies.
Taken together, these observations suggest that GnRH plays an integral role in human implantation, by virtue of its ability to regulate the balance between MMP and TIMP expression in decidual cells.

5.2 Introduction

The decidual cells of the endometrium fulfill paracrine, nutritional, immunoregulatory, and embryoregulatory functions throughout pregnancy (Tabibzadeh et al., 1995, Paria et al., 2002). Decidualization, which begins in the secretory phase and continues into early pregnancy in the human, involves the morphological and biochemical differentiation of the endometrial stroma (Noyes et al., 1950, Kearns et al., 1985). This highly regulated series of developmental events involves the remodeling of the stromal/decidual cell extracellular matrix (ECM). In particular, the interstitial-type ECM of the proliferative endometrium, which is enriched in fibronectin and collagen types I, III, V, and VI is replaced with a basal lamina composed primarily of residual interstitial proteins, laminin, heparin sulfate proteoglycan and collagen type IV (Kislaus et al., 1987. Aplin et al., 1988).

The spatiotemporal expression of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue-specific inhibitor of matrix metalloproteinases (TIMPs), are believed to mediate, at least in part, the cyclic remodeling of the endometrial ECM (Fata et al., 2000, Vu et
al., 2000). In particular, MMP-2 and MMP-9 expression has been associated with the decidualization of human endometrial stromal cells (Paria et al., 2002. Kislaus et al., 1987. Aplin et al., 1988). The composition of the decidual ECM is also modulated by MMPs secreted by the trophoblastic cells of the implanting embryo (Kislaus et al., 1987. Zhang et al., 2002). Of the multiple MMP subtypes identified in the human placenta, the expression of MMP-2 and MMP-9 have been assigned key roles in the invasion of trophoblasts into the underlying maternal decidua (Shimonovitz et al., 1994, Niu et al., 2000). The production of these MMPs by human trophoblasts is down-regulated during the second trimester of pregnancy, paralleling the decline in the invasive capacity of these cells with gestational age (Shimonovitz et al., 1994). TIMP-1, TIMP-2, and TIMP-3, which are constitutively expressed in the human endometrium and placenta, are believed to counterbalance the MMP-mediated degradation of the decidual ECM in both an autocrine and paracrine manner (Niu et al., 2000, Zhang et al., 1999). To date, the factors capable of regulating the balance between MMP/TIMP expression levels at the maternal-fetal interface remain poorly characterized.

GnRH has been detected in the glandular epithelium and stroma of the human endometrium at all stages of the menstrual cycle with maximum levels being expressed in the secretory endometrium and first trimester decidua (Dong et al., 1998, Raga et al., 1998). Furthermore,
high levels of GnRH have been detected in first trimester placental tissues and cells (Rama et al., 2001, Kikkawa et al., 2002). To date, the biological functions of GnRH in these tissues remain poorly understood. However, in view of the close correlation between the expression patterns of GnRH and MMP-2/MMP-9 in the human endometrial stroma and placenta, it is tempting to speculate that this hormone plays a key regulatory role in the MMP-mediated remodeling of the endometrial ECM required for the establishment of pregnancy. In these studies, we have examined the ability of GnRH to regulate MMP-2, MMP-9, and TIMP-1 mRNA levels in primary cultures of human decidual stromal cells.

5.3 Materials and Methods

5.3.1 Tissues

Tissue samples of first trimester decidualis parietalis were obtained from women undergoing elective termination of pregnancy. The use of these tissues was approved by the committee for ethical review of research involving human subjects, University of British Columbia. All patients provided informed written consent.

5.3.2 Cell isolation and culture

Stromal cells were isolated from the decidual tissue samples by enzymatic digestion and mechanical dissociation using a protocol modified from that reported by Shiokawa et al. (1996).
Briefly, the decidual tissue samples were minced and subjected to 0.1% collagenase (type IV, Sigma, St. Louis, MO) and 0.1% hyaluronidase (type I-S) digestion in a shaking water bath at 37 °C for 60 min. The cell digest was then passed through a nylon sieve (38 μm). The isolated glands and any undigested tissue fragments were retained on the sieve, and the eluate containing the stromal cells was collected in a 50-ml tube. The stromal cells were then pelleted by centrifugation at 800 x g for 10 min at room temperature. The cell pellet was washed once with DMEM containing 10% fetal bovine serum before being resuspended and plated in DMEM containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and supplemented with 10% fetal bovine serum. The culture medium was replaced 30 min after plating to reduce epithelial cell contamination. The purity of the decidual stromal cell cultures was determined by immunocytochemical staining for vimentin, cytokeratin, muscle actin, and factor VIII (data not shown). These cellular markers have been used to determine the purity of human endometrial cell cultures (1989). As defined by these criteria, the decidual stromal cell cultures used in these studies contained less than 1% epithelial or vascular cells.

5.3.3 Hormone treatments

The decidual stromal cells were cultured in the presence of increasing concentrations of GnRH
(0, 0.1, 1, 10, or 100 nM) for 24 h before being harvested for total RNA extraction. The hormone concentrations used in these experiments were selected on the basis of previous studies (Raga F et al. 1999). In addition, decidual stromal cell cultures were treated with a combination of GnRH (100 nM) and increasing concentrations (0, 1, 10, or 100 nM) of the GnRH antagonist, antide, for 24 h. Cells treated with vehicle (0.1% ethanol) alone served as a control for these experiments.

5.3.4 Generation of first strand cDNA

Total RNA was prepared from the decidual cell cultures using a RNeasy Mini Kit (QIAGEN, Valencia, CA) and a protocol recommended by the manufacturer. The concentration of total RNA present in each of the extracts was quantified by optical densitometry (260/280 nm) using a Du-64 UV-spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

An aliquot (1 µg) of the total RNA extracts prepared from the decidual stromal cell cultures was reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Amersham Pharmacia Biotech, Oakville, Canada).

5.3.5 Primer design

Nucleotide sequences specific for MMP-2, MMP-9, or TIMP-1, and which also spanned different exons, were identified in the human mRNA sequences (accession nos. XM_165656,
BC006093, and XM_033878, respectively) deposited in GenBank (National Center for Biotechnology Information). Forward and reverse primers corresponding to these DNA sequences were synthesized at the Nucleic Acid and Protein Services Unit, University of British Columbia. Primers specific for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which have been previously described (Tokunaga et al., 1987) were used to quantify and assess the integrity of the total RNA samples. To construct a competitive cDNA fragment, floating primers with a sequence complementary to a short nucleotide sequence between the forward- and reverse-primer binding sites were also generated by attaching the complementary sequence of the binding site to the 3'-end of the original reverse primers specific for MMP-2, MMP-9 or TIMP-1 (Fig. 24). A similar approach has been used to examine the effects of IL-1 on urokinase plasminogen activator (u-PA), soluble u-PA receptor, and plasminogen activator inhibitor-1 mRNA levels in primary cultures of human endometrial stromal cells (Chung et al., 2001). The specific sequences of these primer sets and the expected sizes of the resultant PCR products are listed in Table.

5.3.6 Semiquantitative PCR

Semiquantitative PCR was performed using template cDNA generated from the total RNA extracts prepared from cultures of untreated decidual stromal cells and the primers specific for
MMP-2, MMP-9, or TIMP-1. The PCR conditions were as follows: 1 min at 94 C, 1 min at 57.5 C or 56 C for PAI-1 and u-PA, respectively; and 1.5 min at 72 C followed by a final extension at 72 C for 15 min. The cycles were repeated 20–35 times.

The resultant PCR products of 506, 544, and 369 bp for MMP-2, MMP-9, and TIMP-1, respectively, were separated using gel electrophoresis and visualized by ethidium bromide staining (Fig. 25). To confirm the specificity of the primers, an aliquot of the MMP-2, MMP-9, and TIMP-1 cDNAs generated using total RNA extracts prepared from the decidual cell cultures were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequence analysis.

Southern blot analysis using an aliquot (10 μl) of the MMP-2, MMP-9, and TIMP-1 cDNAs demonstrated that there was a linear relationship between the yield of the PCR products and number of cycles performed. Consequently, 21 cycles for the amplification of GAPDH, 27 cycles for MMP-2, and 30 cycles for MMP-9 and TIMP-1 from first strand cDNA templates generated from decidual stromal cells were used in these studies.

Similarly, PCR using template cDNA generated from the decidual stromal cell cultures and a combination of the regular forward primer specific for MMP-2, MMP-9, or TIMP-1 and the corresponding reverse, floating primer yielded truncated cDNAs with the expected sizes of 294,
395, and 185 bp, respectively. These PCR products were also subcloned into the PCR II vector and subjected to DNA sequence analysis to confirm the specificity of these primer sets.

5.3.7 Quantitative Competitive-PCR (QC-PCR)

The QC-PCR strategy employed in these studies was based upon the competitive coamplification of known amounts of the truncated MMP-2, MMP-9, or TIMP-1 PCR products added to aliquots of first strand cDNA prepared from primary cultures of decidual stromal cells.

To determine the ideal amounts of each template to be added to the reaction mixture, PCR was performed using fixed amount of competitive cDNA (15, 0.1, and 1 pg for MMP-2, MMP-9, and TIMP-1, respectively) and decreasing concentrations of target cDNA (51.2-0.1, 6.4-0.003, and 12.8-0.05 pg for MMP-2, MMP-9, and TIMP-1, respectively), obtained by serial dilution as templates and the corresponding sets of primer. The PCR conditions and number of cycles were performed for the MMP and TIMP primer sets as described above.

An aliquot (10 μl) of the PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining (Fig. 26). The intensity of the ethidium bromide staining of the PCR products was analyzed using UV densitometry (Biometra, Whiteman Co., Gottingen, Germany). Volume counts (mm$^2$) of the PCR products were then determined using the Scion Image computer software (Scion Image Co., Frederick, MD). The intensity of the
ethidium bromide staining of an aliquot (10 µl) of a 100-bp DNA ladder (Life Technologies, Inc.) served as an internal standard.

A standard curve was generated using the logarithmically transformed ratios of target:competitive cDNA plotted against the log amount of target cDNA initially added to the PCR reaction (Fig. 26). This standard curve was highly reproducible and linear. The values obtained from this regression curve \( y = b + mx \) were then used to determine the amounts of cDNA transcripts present in unknown samples. An aliquot of MMP-2 (15 pg/µl), MMP-9 (0.1 pg/µl) or TIMP-1 (1 pg/µl) competitive cDNA was subsequently added to the aliquots of the first strand cDNA generated from the human decidual cells for QC-PCR.

QC-PCR was performed using the MMP-2, MMP-9, or TIMP-1 primer sets, 1 µl of the first-strand cDNA synthesized from each of the cell cultures, and the PCR conditions described above. The ratios of the intensity of ethidium bromide staining of the target:competitive cDNAs were then normalized to the corresponding GAPDH, logarithmically transformed and compared with the values obtained from the standard curve.

5.3.8 Statistical analysis

The absorbance values obtained from the ethidium bromide-stained gels were subjected to statistical analysis using GraphPad Software, Inc. Prism 2 software (San Diego, CA). Statistical
differences between the absorbance values were assessed by the ANOVA. Differences were considered significant for $P$ values no greater than 0.05. Significant differences between the means were determined using Dunnett's test. The results are presented as the mean relative absorbance (± SEM) obtained using five or more different tissue samples.
Figure 24. A representative schematic diagram illustrating the construction of an internal standard cDNA for MMP-2. An internal standard fragment was constructed by deletion of 212-bp fragment from the specific target cDNA to be detected.
Figure 25. Semiquantitative PCR analysis of MMP-2 mRNA levels in primary cultures of human decidual cells. Autoradiograms of Southern blots containing PCR products amplified from first strand cDNA synthesized from decidual stromal cells and primers specific for GAPDH (top) or MMP-2 (bottom) using an increasing number of cycles. The autoradiograms were scanned, and the absorbance values obtained for each PCR product were plotted against the corresponding number of cycles. The observed linear relationships between the yield of these distinct PCR products and the number of amplification cycles are shown in the graphs below. A linear relationship between the yield of PCR product and the number of amplification cycles using template cDNA synthesized from decidual stromal cells was also observed for MMP-9 and TIMP-1 (data not shown).
**A**

\[ y = 0.1192x - 0.0512 \]

\[ \text{FF} = 0.9627 \]

Native MMP2 cDNA (pg)

**B**

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</table>

Mutant cDNA (pg)  400  200  100  50  25  12.5  6.25  3.12  1.56
Figure 26. Preparation of a standard curve for the quantitative competitive PCR analysis of MMP-2 mRNA levels in decidual stromal cells. A, Photomicrograph of an ethidium bromide stained gel containing PCR products generated using an increasing amount of native cDNA and the two primer sets specific for MMP-2. The sizes of the resultant PCR products relative to a 100-bp ladder (MW) are indicated. The intensity of the ethidium bromide staining of the target and competitive PCR products was determined by UV densitometry. The observed linear relationship between the logarithmically transformed ratios of target:competitive cDNA and the amount of native cDNA added to each PCR is shown in the graph below. B, Photomicrograph of an ethidium bromide gel containing PCR products generated using a fixed amount of template cDNA (1 μl) synthesized from decidual stromal cells and a decreasing amount of the 294-bp internal standard cDNA for MMP-2 obtained by serial dilutions. The intensity of the ethidium bromide staining of the target and competitive PCR products was determined by UV densitometry. The linear relationship between the logarithmically transformed ratios of target:internal standard cDNAs and the amount of mutant cDNA added to the initial PCR is shown in the graph below. The point at which these two graphs intercept (12.5–25 pg) indicates the amount of MMP-2 cDNA that can be generated from 1 μg of total RNA extracted from isolated human decidual stromal cells. Standard curves for MMP-9 and TIMP-1 were also generated using the same approach (data not shown).
5.4 Results

5.4.1 Dose-dependent effects of GnRH on MMP-2, MMP-9, and TIMP-1 mRNA levels in human decidual stromal cells

MMP-2, MMP-9, and TIMP-1 mRNA transcripts were detected in all of the total RNA extracts prepared from the isolated human decidual stromal cells (Fig. 27). The addition of vehicle (0.1% ethanol) had no significant effect on the levels of the MMP-2, MMP-9, or TIMP-1 mRNA transcripts in these primary cell cultures (data not shown). In contrast, GnRH increased MMP-2 and MMP-9 mRNA levels in isolated decidual stromal cells in a dose-dependent manner (Fig. 28, A and B). Maximum levels of MMP-2 mRNA levels were observed in decidual cells cultured in the presence of 1 nM GnRH. The addition of higher concentrations of GnRH to the culture medium had no further effect on the levels of the MMP-2 mRNA transcript present in these cells. A significant increase in MMP-9 mRNA levels was only observed in decidual stromal cells cultured in the presence of 100 nM GnRH. In contrast, GnRH had no significant effect on the levels of the mRNA transcripts encoding TIMP-1 present in these primary cell cultures at any of the concentrations used in these studies.
Figure 27. Photomicrograph of an agarose gel containing PCR products generated using template cDNA synthesized from isolated human decidual stromal cells and primers specific for GAPDH (lane 1), MMP-2 (lane 2), TIMP-1 (lane 3), and MMP-9 (lane 4). The expected sizes of the distinct PCR products; GAPDH (373 bp), MMP-2 (506 bp), MMP-9 (544 bp), and TIMP-1 (379 bp) are indicated relative to a 100-bp DNA ladder (lane MW). Similar results were obtained using different cell cultures on five independent occasions.
**Figure 28.** QC-PCR analysis of total RNA extracts prepared from decidual cells cultured in the presence of increasing concentrations of GnRH. Representative photomicrographs of ethidium bromide-stained gels containing PCR products generated using template cDNA synthesized from decidual cells cultured in the presence of 0, 0.1, 1, 10, or 100 nM (lanes 1–5, respectively) and the primer sets specific for MMP-2 (A), MMP-9 (B), or TIMP-1 (C). Each sample was coamplified in the presence of a defined amount of internal standard cDNA. The sizes of the resultant target and competitive PCR products relative to a 100-bp ladder (lane MW) are marked to the right of the photomicrograph. The intensity of the ethidium bromide staining of the PCR products was determined by UV densitometry and the absorbance values obtained for each PCR normalized to the values obtained for the corresponding GAPDH (lower panels). These normalized values were used to calculate the ratio of target to internal standard cDNA for each QC-PCR. The results derived from this analysis as well as from four other studies (data not shown) are represented (mean ± SEM, n = 5) in the bar graphs below (a, *P* < 0.05 vs. untreated control).
5.4.2 Antide inhibits the stimulatory effects of GnRH on MMP-2 and MMP-9 mRNA levels in primary cultures of human decidual cells

The GnRH antagonist, antide, inhibited the stimulatory effects of GnRH on MMP-2 and MMP-9 mRNA levels in isolated human stromal decidual cells in a dose-dependent manner (Fig. 29, A and B). In contrast, antide had no significant effect on TIMP-1 mRNA levels in decidual cells cultured in the presence or absence of GnRH (Fig. 29C).
Figure 29. QC-PCR analysis of total RNA extracts prepared from decidual cells cultured in the presence or absence of GnRH and increasing concentrations of antide. Representative photomicrographs of ethidium bromide-stained gels containing PCR products generated using template cDNA synthesized from untreated decidual cells (lane 1) or cells cultured in the presence of 100 nM GnRH and 0, 1, 10, or 100 nM antide (lanes 2–5, respectively) and the primer sets specific for MMP-2 (A), MMP-9 (B), or TIMP-1 (C). Each sample was coamplified in the presence of a defined amount of internal standard cDNA. The sizes of each of the target and competitive PCR products relative to a 100-bp ladder (lane MW) are marked to the right of the photomicrographs. The intensity of the ethidium bromide staining of the PCR products was determined by UV densitometry and the absorbance values obtained for each normalized to the values obtained for the corresponding GAPDH. These normalized values were used to calculate the ratio of target to internal standard cDNA for each QC-PCR. The results derived from this analysis as well as from four other studies (data not shown) are represented (mean ± SEM n = 5) in the bar graphs below (a, $P < 0.05$ vs. untreated control).
5.5 Discussion

MMP-2 and MMP-9 were readily detectable in stromal cells isolated from first trimester human decidual tissues. Similarly, MMP-2 and MMP-9 activity has been detected in human decidual tissues throughout pregnancy (Xu et al., 2001). The expression of these two MMPs is also highly regulated during the decidualization of the rat uterus (Woessner et al., 1996, Rechtman et al., 1999). Furthermore, the administration of the MMP inhibitor doxycycline to these animals during early pregnancy delayed decidualization but did not inhibit implantation (Rechtman et al., 1999). Taken together, these observations suggest that MMP-2 and MMP-9 play key roles in the terminal differentiation of endometrial stomal cells into decidua. These MMPs may mediate decidualization not only via their ability to remodel the endometrial ECM but also in the release and/or activation of growth factors, and vasoactive factors (Rechtman et al., 1999).

The spatiotemporal expression and/or activity of MMPs in the human endometrium during the menstrual cycle suggests that gonadal steroids are key regulators of these proteases (Fata et al., 2000, Zhang et al., 2002). Progesterone has been shown to be capable of decreasing MMP-2 and MMP-9 expression levels in human endometrial stromal cells in vitro (Schatz et al., 1999). However, as MMP-2 and MMP-9 are readily detectable in the decidua, when progesterone levels are elevated, it is likely in addition to gonadal steroids, MMP expression levels in the
endometrium are modulated by localized, regulatory factors. These regulatory factors may act in an autocrine and/or paracrine manner. For example, recent studies indicate that activation of MMP-2 in the endometrial stroma involves as yet unidentified, soluble factor(s) that are secreted by glandular epithelial cells (Goffin et al., 2002). We have determined that GnRH, which is expressed by both the glandular epithelium and stroma of the endometrium (Raga et al., 1998), is capable of regulating MMP expression levels in decidual stromal cells in vitro. In addition, GnRH secreted by subpopulations of human trophoblasts at the maternal fetal interface is also likely to regulate MMP activity in the decidua.

Increasing concentrations of GnRH had differential effects on MMP-2 and MMP-9 mRNA levels in decidual stromal cells. The decrease in MMP-2 mRNA levels in the cells cultured in the presence of the highest concentrations of GnRH used in these studies may be attributed to desensitization, a biological phenomenon observed in the GnRH-stimulated secretion of gonadotropins by pituitary cells (McArdle et al., 2002). Furthermore, as MMP-9 mRNA levels were increased in these cell cultures, it is tempting to speculate that the regulatory effects of GnRH on the expression of these two MMPs in the human decidua is mediated by distinct intracellular signaling pathways. Recent studies indicate that GnRH is capable of activating the protein kinase A, protein kinase C, and/or MAPK signaling pathways in a wide variety of human
cells (Cheng et al., 2000). Although the importance of one or more of these signaling pathways in the GnRH-mediated expression of MMPs in human decidual cells has yet to be elucidated, at least one response element for the downstream effector of the MAPK cascade, activator protein-1, has been detected in the promoter region of the human gene encoding MMP-9 (Matrisian et al., 1994). In contrast, the MMP-2 promoter appears to be devoid of such response elements (Matrisian et al., 1994).

Human endometrial stromal cells cultured in the presence of gonadal steroids undergo morphological and biochemical differentiation processes that mimic decidualization (Irwin et al., 1989). However, Raga et al. (1999) failed to detect MMP-9 mRNA transcripts in these primary cell cultures, a predominant MMP subtype present in human decidual tissues (Raga et al., 1999). Furthermore, in contrast to our findings, GnRH agonists were shown to increase the levels of TIMP-1 and TIMP-3 but have no effect on MMP-9 mRNA in these primary cell cultures (Raga et al., 1999). These discrepancies may be attributed to differences between the repertoire of MMP/TIMPs expressed by endometrial stromal cells that have undergone decidualization in vivo or in vitro and/or the culture conditions used in these two studies. In particular, the pharmacological doses of gonadal steroids required to induce decidualization in human
endometrial stromal cells in vitro is likely to have profound effects on MMP and TIMP expression levels in these primary cell cultures.

TIMP-1 is constitutively expressed in the human endometrium throughout the menstrual cycle and pregnancy (Irwin et al., 1989). As TIMPs inactivate MMP activity by binding their active forms with a 1:1 stoichiometry (Nagase et al., 1999), the localized remodeling of the endometrial ECM will be dependent on the balance between the expression levels of MMPs and TIMPs in this dynamic tissue. We have determined that GnRH regulates MMP-2/MMP-9 but not TIMP-1 mRNA levels in primary cultures of human decidual stromal cells. Similarly, epidermal growth factor and basic fibroblast growth factor were capable of increasing MMP-3, MMP-9, and MMP-13 but had no significant effect on TIMP-1, TIMP-2, or TIMP-3 in rat endometrial stromal cells undergoing decidualization in vitro (Nuttall et al., 2000). In contrast, IL-1 increased MMP-9 and decreased TIMP-1 and TIMP-3 mRNA levels in primary cultures of human endometrial stromal cells, whereas TGF-β decreased the levels of MMP-9 and increased TIMP-1 and TIMP-2 mRNA levels in these primary cell cultures (Huang et al., 1998).

Low binding affinity/high capacity binding sites for GnRH have been observed in the endometrium and endometrial carcinomas suggesting that this hormone plays a direct, regulatory role in the remodeling processes that occur in this dynamic tissue under normal and
pathological conditions (Emons et al., 1993). Furthermore, immunization of immature mice with antibodies directed against a peptide corresponding to amino acids 5–17 of the murine GnRH receptor (GnRHR) resulted in the specific regression/inhibition of endometrial cell proliferation (Asirvatham et al., 1994). Antide was capable of inhibiting the GnRH-mediated increase MMP-2 and MMP-9 mRNA levels in isolated decidual stromal cells in a dose-dependent manner suggesting that these stimulatory effects are mediated by GnRH interacting with its receptor (GnRHR). However, previous studies have failed to detect significant levels of GnRHR in the human endometrium during the secretory phase of the menstrual cycle and decidua of early pregnancy (Raga et al., 1999, Ikeda et al., 1997). Recently, a gene encoding a second receptor for GnRH (GnRHR II) has been identified in the human genome (Millar et al., 2001, Neill et al., 2002). Although a full-length mRNA transcript encoding this second form of human GnRHR has not been isolated, GnRHR II mRNA transcripts have been detected in human uterine tissues and endometrial cancer lines (Neill et al., 2002, Grundker et al., 2002).

In summary, we have determined that GnRH is capable of increasing MMP-2 and MMP-9, but not TIMP-1 mRNA levels in primary cultures of stromal cells isolated from first trimester human decidual tissues. The GnRH antagonist, antide, was capable of inhibiting the stimulatory effects of GnRH on MMP-2 and MMP-9 mRNA levels in these primary cell cultures.
Collectively, these observations suggest that GnRH is a key regulator of the MMP-mediated ECM remodeling events that occur in the stroma of the human endometrium in preparation for pregnancy.
PART VI Regulatory Effects of GnRH I and GnRH II on MMP-2, MMP-9, and TIMP-1 Expression in Human Extravillous Cytotrophoblasts.

6.1 ABSTRACT

An intricate balance between the production of matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue specific inhibitors of matrix metalloproteinases (TIMPs), modulates the over-all proteolytic activity of trophoblasts during human implantation. In these studies, we have examined the ability of the classical Gonadotropin-Releasing Hormone (GnRH I) and the second form of GnRH (GnRH II) to regulate MMP-2, MMP-9 and TIMP-1 mRNA and protein levels in extravillous cytotrophoblasts propagated from first trimester chorionic villi. GnRH I and GnRH II were found to increase MMP-2 and MMP-9 mRNA and protein levels in these primary cell cultures in a dose- and time-dependent manner using quantitative competitive-PCR and ELISA. In contrast, these two hormones decreased trophoblastic TIMP-1 mRNA and protein levels. Cetrorelix, a GnRH receptor antagonist, inhibited the regulatory effects of GnRH I, but not GnRH II on MMP-2, MMP-9 and TIMP-1 expression in these cells. Collectively, these observations suggest that MMP-2, MMP-9 and TIMP-1 expression in human trophoblasts are differentially regulated by GnRH I and GnRH II, possibly via distinct receptor-mediated intracellular signaling pathways.
6.2 INTRODUCTION

The invasion of embryonic trophoblasts into the maternal decidua and uterine vasculature, which increases blood flow to the placenta and ensures an adequate supply of nutrients and oxygen to the growing fetus, is a critical step in human pregnancy (Pijnenborg et al. 1980, 1983, Aplin et al., 1991). This highly regulated developmental process is mediated, at least in part, by the spatiotemporal expression of matrix metalloproteinases (MMPs) in both the decidual cells and the distinct subpopulations of trophoblasts present at the maternal-fetal interface (Fata et al., 2000 Vu et al., 2000).

Of the multiple MMPs produced by the human placenta (Graham et al., 1993, Tarrade et al., 2002), MMP-2 and MMP-9 have been assigned key roles in promoting the invasive capacity of cytotrophoblasts. In particular, MMP-2 and MMP-9 are expressed primarily by extravillous cytotrophoblasts (EVTs) in vivo and in vitro. The production of these two MMPs by the placenta is down-regulated during the third trimester of pregnancy, paralleling the decline in trophoblast invasiveness associated with gestational age (Polette et al., 1994, Isaka et al., 2003, Shimonovitz et al., 1994). In addition, function-perturbing antibodies specific for MMP-9 or MMP-2 have been shown to be capable of reducing the invasive capacity of EVT in vitro (Isaka et al., 2003, Librach et al., 1993). Reduced MMP-9 activity has also been reported in primary cultures of cytotrophoblasts isolated from placentae diagnosed with preeclampsia.
(Graham et al., 1996), a disease in which trophoblast invasion into the maternal decidua and vasculature is believed to be compromised (Robertson et al., 1986, Redline et al., 1995).

MMPs are synthesized as latent precursors that must be cleaved following secretion in order to become activated (Nagase et al., 1999). The activity of MMPs are further regulated by the secretion of specific tissue inhibitors of MMPs (TIMPs) (Gomez et al., 1997). TIMP-1, -2, and -3 are produced by the human placenta and decidua, suggesting an autocrine and paracrine regulation of the MMP-mediated invasion of trophoblasts (Graham et al., 1991, Hurskainen et al., 1996, Ruck et al., 1996). In addition, TIMP-1 has been shown to inhibit the invasive capacity of primary cultures EVTs indicating that an intricate balance between the production of proteases and their inhibitors modulates the over-all proteolytic activity of trophoblasts in vivo and in vitro.

We have recently determined that gonadotropin-releasing hormone (GnRH) is capable of increasing MMP-2 and MMP-9 mRNA levels in stromal cells isolated from first trimester decidual tissues (Chou et al., 2003). This hormone has also been shown to decrease the levels of TIMP-1 and TIMP-3 present in human endometrial stromal cells allowed to undergo steroid-mediated decidualisation in vitro (Raga et al., 1999). Taken together, these observations suggest that GnRH plays a key role in human implantation and placentation by regulating MMP activity at the maternal-fetal interface. In these studies, we have examined the ability of GnRH
(GnRH I) and the second form of mammalian GnRH (GnRH II), both of which are expressed by the human placenta and endometrium (Neill et al., 2001, Siler-Khodr et al., 1978, Pragar et al., 1992), to regulate MMP-2, MMP-9 and TIMP-1 mRNA and protein levels in primary cultures of EVTs propagated from first trimester placental tissues in a dose-, and time-dependent manner.
6.3 MATERIALS AND METHODS

6.3.1 Tissues

Tissue samples of first trimester placenta were obtained from women undergoing elective termination of pregnancy. The use of these tissues was approved by the committee for ethical review of research involving human subjects, University of British Columbia. All patients provided informed written consent.

6.3.2 Cell Isolation and Culture

EVTs were propagated from first trimester placental tissue explants as described by Graham et al. (1992). Briefly, chorionic villi were washed thoroughly in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Burlington, ON) containing penicillin and streptomycin (100 IU/ml and 100 ug/ml, respectively). The villi were minced finely and plated in 25cm² tissue culture flasks (Becton Dickinson, Franklin, NJ) containing DMEM supplemented with antibiotics and 10% heated-inactivated fetal bovine serum (FBS; Gibco BRL). The fragments of chorionic villi were allowed to adhere for 2-3 days, after which, any non-adherent material was removed. These tissue explants were cultured for a further 10-14 days with the culture medium being replaced every 2 days. EVTs were separated from the villous explants by a brief (2-3 min) trypsin digestion (0.125% v/v trypsin-EDTA/Ca²⁺-, Mg²⁺-free PBS) at 37°C and plated in 60 mm² culture dishes (Becton Dickinson) containing DMEM supplemented with antibiotics and
10% FBS.

The purity of the EVT cultures was determined by immunostaining with a monoclonal antibody directed against cytokeratin 8 and 18 (Becton Dickinson) according to the methods of MacCalman et al. (1996). Only cell cultures that exhibited 100% immunostaining for cytokeratin were included in these studies.

All studies were performed using EVTs (passage 2) plated in 60 mm² culture dishes at a density of 1x 10⁶ cells (Becton Dickinson) and grown to 80% confluency. Twenty four h before each hormone treatment, FBS was removed from the culture medium.

6.3.3 Hormone Treatments

EVTs were cultured in the presence of increasing concentrations of GnRH I or GnRH II (0, 10 pM, 1 nM or 100 nM) for 24 h or a fixed concentration of GnRH I or GnRH II (100 nM) for 0, 3, 6, 12, 24 or 48 h. In addition, cultures of EVTs were treated with GnRH I or GnRH II (100 nM) alone or in combination with Cetrorelix (100nM), a GnRH I antagonist, for 24h. Cells treated with vehicle (0.1% ethanol) served as a control for all of these experiments.

6.3.4 Primer Design

Nucleotide sequences specific for MMP-2, MMP-9 or TIMP-1 and which also spanned different exons, were identified in the human mRNA sequences deposited in GenBank (National
Center for Biotechnology Information, Bethesda, MD). Forward and reverse primers corresponding to these DNA sequences and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were used to quantify and assess the integrity of the total RNA samples, were synthesized at the Nucleic Acid and Protein Synthesis (NAPS) Unit, University of British Columbia. To construct internal standard cDNA fragments, floating primers with a sequence complementary to a short nucleotide sequence present in the expected PCR products were generated by attaching the complementary sequences of these binding sites to the 3'-end of the original reverse primers specific for MMP-2, MMP-9 or TIMP-1. We have recently used this approach to examine the effects of GnRH I on MMP-2, MMP-9 or TIMP-1 mRNA levels in primary cultures of human endometrial stromal cells (Chou CS et al. 2003). The specific sequences of these primer sets and the expected sizes of the resultant PCR products are listed in Table 1.

6.3.5 RNA Preparation and RT-PCR

Total RNA was prepared from the EVT cultures using a RNeasy Mini Kit (Qiagen, Inc, CA) using a protocol recommended by the manufacturer. The concentration of total RNA present in each of these extracts was quantified by optical densitometry (260/280nm) using a Du-64 UV-spectrophotometer. An aliquot (1μg) of the total RNA extracts prepared from the
EVTs was reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Oakville, Canada).

PCR was performed using template cDNA generated from total RNA extracts prepared from EVT cultures and the primers sets specific for MMP-2, MMP-9 or TIMP-1. The PCR conditions were as follows: 1 min at 94°C, 1 min at 56°C, 59°C or 55°C for MMP-2, MMP-9 or TIMP-1, respectively; and 1.5 mins at 72°C followed by a final extension at 72°C for 15 min. The cycles were repeated 20-35 times. A linear relationship between the yield of the PCR products and number of cycles performed was observed after 25 cycles for MMP-2, 30 cycles for MMP-9 and 27 cycles for TIMP-1 (data not shown).

The resultant PCR products for MMP-2, MMP-9 or TIMP-1 were separated using gel electrophoresis and visualised by ethidium bromide staining (data not shown). An aliquot of these MMP-2, MMP-9 or TIMP-1 PCR products were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and selected clones subjected to DNA sequence analysis to confirm the specificity of the primers. These clones were also used to generate target or internal standard MMP-2, MMP-9 or TIMP-1 cDNA fragments by standard molecular biology techniques.

6.3.6 Quantitative Competitive -Polymerase Chain Reaction) QC-PCR
The QC-PCR strategy employed in these studies is based upon the competitive co-amplification of a known amount of an internal standard specific for MMP-2, MMP-9 or TIMP-1 added to aliquots of the first strand cDNA prepared from our primary cultures of EVTs (22,29,30).

To determine the optimal amounts of the internal standards to be used in the QC-PCR analysis, PCR reaction mixtures containing a fixed amounts of the target MMP-2, MMP-9 or TIMP-1 cDNAs (1μl) and increasing concentrations of the corresponding internal standard cDNAs (0.3125-40 pg/μl for MMP-2, 0.075-10 pg/μl for MMP-9 or 3.125-400 pg/μl TIMP-1, respectively) were prepared. PCR was then performed using these cDNA mixtures and the distinct MMP-2, MMP-9 or TIMP-1 primer sets under the optimized conditions described above.

An aliquot (10 μl) of the resultant MMP-2, MMP-9 or TIMP-1 PCR products were separated by electrophoresis in a 1% agarose gel and visualised by ethidium bromide staining (Fig 30). The intensity of the ethidium bromide staining of the PCR products was analysed using UV densitometry (Biometra, Whiteman Co., Gottigen, Germany). Volume counts (mm²) of the scanned PCR products were then determined using the Scion Image computer software (Scion Image Co, Frederick, MD). The absorbance values obtained for each of the target and corresponding internal standard cDNAs generated by PCR were plotted against the amount of

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internal standard initially added to the reaction mixtures. The point of interception on these line
graphs was taken as the optimal amount of internal standard to be used in the QC-PCR analysis
(Fig. 30). Based upon these observations, MMP-2, MMP-9 or TIMP-1 internal standard
cDNAs were added to aliquots of the first strand cDNA generated from the EVTs to be used in
the QC-PCR analysis at concentrations of 5 pg/μl, 1.25 pg/μl or 50 pg/μl, respectively.

QC-PCR was performed using an aliquot (1 μl) of the first strand cDNA synthesized from
the EVTs containing the optimized amount of the corresponding internal standard cDNA and
the MMP-2, MMP-9 or TIMP-1 primer sets under the PCR conditions described above. The
ratios of the intensity of ethidium bromide staining of the resultant target: internal standard PCR
products were then determined as described above.
6.3.7 ELISA

MMP-2 or MMP-9 activity and TIMP-1 expression levels were measured in the conditioned medium of the EVTs by ELISA using commercially available kits (Amersham pharmacia biotech, Piscataway, NY). MMP-2 or MMP-9 activity and TIMP-1 expression levels were detected in the conditioned culture medium with a mean intra-assay and inter-assay coefficient of variation of 7% and 16.9% for MMP-2, 4.3% and 20.2% for MMP-9, and 8.9% and 13.1% for TIMP-1, respectively. All samples were assayed in duplicate.

6.3.8 Statistical Analysis

The absorbance values obtained from the ethidium bromide stained gels were subjected to statistical analysis using GraphPad Prism 2 computer software (San Diego, CA, USA). 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 \( \pm \) the standard error of the mean (SEM) obtained using \( \geq 5 \) different cell preparations.

Statistical differences between the dose- or time-dependent effects of GnRH I or GnRH II on MMP-2, MMP-9 activity or TIMP-1 expression levels present in the conditioned media of the EVT cultures were assessed by ANOVA followed by Dunnett’s test. The results are
presented as the mean protein expression levels ± SEM obtained using cultures propagated from 
≥5 different placental tissues.
A.

![A Diagram Showing Target and Internal Standard cDNA](image)

- Target cDNA
- Internal standard cDNA

**Volume count (mm²)**

<table>
<thead>
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<th>Volume count (mm²)</th>
<th>15000</th>
<th>10000</th>
<th>5000</th>
<th>0</th>
</tr>
</thead>
</table>

**Internal standard (pg/ul)**

- 0.3125
- 0.625
- 1.25
- 2.5
- 5
- 10
- 20
- 40

B.

![B Diagram Showing Target and Internal Standard](image)

- Target cDNA
- Internal standard

**Volume count (mm²)**

<table>
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<th>8000</th>
<th>6000</th>
<th>4000</th>
<th>2000</th>
<th>0</th>
</tr>
</thead>
</table>

**Internal standard (pg/ul)**

- 0.075
- 0.15
- 0.3125
- 0.625
- 1.25
- 2.5
- 5
- 10

C.

![C Diagram Showing Target and Internal Standard](image)

- Target cDNA
- Internal standard

**Volume count (mm²)**

<table>
<thead>
<tr>
<th>Volume count (mm²)</th>
<th>15000</th>
<th>10000</th>
<th>5000</th>
<th>0</th>
</tr>
</thead>
</table>

**Internal standard (pg/ul)**

- 3.125
- 6.25
- 12.5
- 25
- 50
- 100
- 200
- 400
Figure 30. Determination of the optimal amounts of internal standard MMP-2 (A), MMP-9 (B) or TIMP-1 (C) cDNAs to be added to the QC-PCR reaction mixtures. Photomicrographs of ethidium bromide stained gels containing PCR products generated using a fixed amount of target cDNA and increasing amounts of the corresponding internal standard (upper panels). The sizes of the resultant PCR products relative to a 100bp ladder (MW) are indicated.

The intensity of the ethidium bromide staining of these PCR products was determined by UV densitometry and the resultant absorbance values of the ethidium plotted against the amount of internal standard added to each PCR reactions in the line graphs (lower panels).
6.4 RESULTS

6.4.1 GnRH I and GnRH II increase MMP-2 and MMP-9 mRNA and protein levels in EVT in a dose-dependent manner.

MMP-2 and MMP-9 mRNA transcripts and protein were detected in all of the EVT cultures examined in these studies (Figs 31 and 32).

GnRH I increased MMP-2 and MMP-9 mRNA and protein levels in primary cultures of EVTs in a dose-dependent manner (Fig. 31). A significant increase in the levels of the mRNA transcripts encoding these 2 MMP subtypes was only detected in EVTs cultured in the presence of the highest concentration of GnRH I (100 nM) examined in these studies. However, a significant increase in the expression of these 2 MMP subtypes was first detected in EVTs cultured in the presence of lower concentrations of this hormone (1nM).

GnRH II significantly increased MMP-2 mRNA and protein levels in these primary cell cultures at all of the hormone concentrations examined in these studies (Fig. 32). In contrast, increased MMP-9 mRNA and protein levels were only detected in EVTs cultured in the presence of 100 nM of this hormone.
A. 

![Image of gel electrophoresis with bands at 505bp and 294bp, and a bar graph showing target/standard cDNA levels for GnRH I at 0, 10pM, 1nM, and 100nM.]

B. 

![Image of gel electrophoresis with bands at 544bp and 395bp, and a bar graph showing target/internal standard cDNA levels for GnRH I at 0, 10pM, 1nM, and 100nM.]

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C. MMP2

\[ \text{MMP2 (ng/mg protein)} \]

GnRH I 0 10 pM 1 nM 100 nM

D. MMP9

\[ \text{MMP9 (ng/mg protein)} \]

GnRH I 0 10 pM 1 nM 100 nM
Figure 31. MMP-2 and MMP-9 mRNA and protein expression levels in EVTs cultured in the presence of increasing concentrations of GnRH I. Panels A and B: Representative photomicrographs of ethidium bromide stained gels containing QC-PCR products generated using template cDNA synthesised from EVTs cultured in the presence of 0, 10 pM, 1 nM or 100 nM GnRH I (lanes 1-4, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked on the right hand-side of the photomicrograph. The intensity of the ethidium bromide staining of the PCR products was determined by UV densitometry and the resultant absorbance values used to calculate the ratio of target to internal standard cDNA for each QC-PCR reaction. The results derived from this analysis as well as from four other independent studies (data not shown) are represented (mean ± SEM n=5) in the bar graphs below (a, P<0.001 vs. untreated control).

Panels C and D: Analysis of the levels of active MMP-2 (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. One milligram of protein from conditioned medium was used in each reaction. Data are shown as the means of five independent assays ± SEM (a, P<0.001; b, P<0.05 vs untreated control) in the bar graphs.
C.

![Bar chart showing MMP2 levels (ng/mg protein) for different GnRH II concentrations: 0, 10 pM, 1 nM, and 100 nM.](image)

D.

![Bar chart showing MMP9 levels (ng/mg protein) for different GnRH II concentrations: 0, 10 pM, 1 nM, and 100 nM.](image)
Figure 32. MMP-2 and MMP-9 mRNA and protein levels in EVTs cultured in the presence of increasing concentrations of GnRH II. Panels A and B: Representative photomicrographs of ethidium bromide stained gels containing QC-PCR products generated using template cDNA synthesised from EVTs cultured in the presence of 0, 10 pM, 1 nM or 100 nM GnRH II (lanes 1-4, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control).

Panels C and D: Analysis of the levels of active MMP-2 (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs untreated control) in the bar graphs.
6.4.2 GnRH I and GnRH II increase MMP-2 and MMP-9 mRNA and protein levels in EVT
in a time-dependent manner.

A significant increase in MMP-2 mRNA levels was detected in EVT cultured in the presence of GnRH I for 6 h with maximum levels of this mRNA transcript being detected after 24 h of treatment (Fig. 33). However, a significant increase in the levels of active MMP-2 present in the conditioned medium of these cells was only detected after 12 h of culture under these experimental conditions. A significant increase in MMP-9 mRNA and protein levels was also observed in EVT cultured in the presence of GnRH I for 12 h and 24 h, respectively. There was a significant decline in the mRNA and protein levels of both these MMP subtypes after 48 h of culture in the presence of GnRH I.

GnRH II caused a significant increase in MMP-2 mRNA levels in EVT cultured in the presence of this hormone after 24 h (Fig. 34). MMP-2 protein levels were also increased after 12 h of treatment with maximum expression levels being detected in the conditioned medium of EVT cultured in the presence of GnRH II for 24 h. Similarly, MMP-9 mRNA and protein levels were significantly higher in EVT cultured in the presence of GnRH II for 12 h and continued to increase until 24 h. A significant decline in the mRNA and protein levels of both these MMP subtypes was observed in EVT cultured in the presence of this hormone for 48 h.
The addition of vehicle to the culture medium had no significant effect on MMP-2, MMP-9 mRNA or protein levels in these primary cell cultures at any of the time points examined in these studies (data not shown).
Figure 33: Time-dependent effects of GnRH I on MMP-2 or MMP-9 mRNA and protein levels in EVTs. Panels A and B: QC-PCR analysis of MMP-2 (Panel A) or MMP-9 (Panel B) mRNA levels in EVTs cultured in the presence of GnRH I (100 nM) for 0, 3, 6, 12, 24 or 48 h (lanes 1-6, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control) Panels C and D: Analysis of the levels of active MMP-2 (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM (a, P<0.001 vs untreated control) in the line graphs.
Figure 34: Time-dependent effects of GnRH II on MMP-2 or MMP-9 mRNA and protein levels in EVTs. Panels A and B: QC-PCR analysis of MMP-2 or (Panel A) or MMP-9 (Panel B) mRNA levels in EVTs cultured in the presence of GnRH II (100 nM) for 0, 3, 6, 12, 24 or 48 h (lanes 1-6, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control)

Panels C and D: Analysis of the levels of active MMP-2 or (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM (a, P<0.001; b, P<0.05 vs. untreated control) in the line graphs.
6.4.3 GnRH-I and GnRH-II decrease TIMP-1 mRNA and protein levels in EVTs in a dose- and time-dependent manner

TIMP-1 mRNA and protein were detected in all of the EVT cultures examined in these studies.

GnRH-I and GnRH-II decreased TIMP-1 mRNA and protein levels in a dose-dependent manner. A significant decrease in TIMP-1 mRNA and protein levels was only observed in EVTs cultured in the presence of the higher concentrations of GnRH I (1 and 100 nM) used in these studies (Fig 35). Similarly, only the higher concentrations of GnRH II (100 nM) were capable of significantly decreasing TIMP-1 mRNA levels in these primary cell cultures. In contrast, GnRH II was capable of decreasing the TIMP-1 protein levels at all of the hormone concentrations examined in these studies.

A significant decrease in TIMP-1 mRNA and protein levels was observed in EVTs cultured in the presence of either GnRH I or GnRH II after 12 h which was followed by a progressive and significant increase in TIMP-1 expression levels in these cells until the termination of these studies at 48 h of treatment (Fig 36).

The addition of vehicle to the culture medium had no significant effect on TIMP-I mRNA or protein levels in these primary cell cultures at any of the time points examined in these studies (data not shown).
A.

Target/internal standard cDNA

GnRH I 0 10 pM 1 nM 100 nM

B.

Target/internal standard cDNA

GnRH II 0 10 pM 1 nM 100 nM
Figure 35: TIMP-1 mRNA and protein levels in EVTs cultured in the presence of increasing concentrations of GnRH I or GnRH II. Panels A and B: QC-PCR analysis of TIMP-1 mRNA levels in EVTs cultured in the presence of 0, 10 pM, 1 nM or 100 nM GnRH I or GnRH II (lanes 1-4, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control)

Panels C and D: Analysis of TIMP-1 expression in conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM (a, P<0.001; b, P<0.05 vs. untreated control) in the bar graphs.
Figure 36: Time-dependent effects of GnRH I or GnRH II on TIMP-1 mRNA and protein levels in EVTs. Panels A and B: QC-PCR analysis of TIMP-1 mRNA levels in EVTs cultured in 100 nM of GnRH I (Panel A) or GnRH II (Panel B) for 0, 3, 6, 12, 24 or 48 h (lanes 1-6, respectively). The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001; b, P<0.05 vs. untreated control). Panels C and D: ELISA analysis of TIMP-1 level in conditioned medium of these EVT cultures treated with 100 nM of GnRH I (Panel C) or GnRH II (Panel D) for 0, 3, 6, 12, 24 or 48 h, respectively. Data are shown as the means of five independent assays± SEM (a, P<0.001 vs untreated control) in the line graphs.
6.4.4 Cetrorelix inhibits the regulatory effects of GnRH I but not GnRH II on MMP-2, MMP-9 and TIMP-1 mRNA and protein levels in EVTs

Cetrorelix, a GnRHR antagonist, was capable of inhibiting the stimulatory effects of GnRH I but not GnRH II on MMP-2 and MMP-9 mRNA and protein levels in EVTs (Figs 37 and 38, respectively). Similarly, Cetrorelix inhibited the decrease in TIMP-1 mRNA and protein levels in EVTs cultured in the presence of GnRH I but had no significant effect on the GnRH II-mediated down-regulation of TIMP-1 expression levels in these cells (Fig. 39).
A.

![Image A with DNA gel and bar chart showing target/internal standard cDNA levels for GnRH I and Cetrorelix at 0, 100 nM, and 100 nM doses.](image)

B.

![Image B with DNA gel and bar chart showing target/internal standard cDNA levels for GnRH I and Cetrorelix at 0, 100 nM, and 100 nM doses.](image)
Figure 37: Effects of Cetrorelix on MMP-2 or MMP-9 mRNA and protein levels in EVTs cultured in the presence of GnRH I. Panels A and B: QC-PCR analysis of MMP-2 (Panel A) or MMP-9 (Panel B) mRNA levels in untreated EVTs (lane 1), or cells cultured in the presence of a fixed amount of GnRH I (100 nM) alone (lane 2) or in combination with Cetrorelix (100 nM) (lane 3). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001 vs. treatment with GnRH I alone). Panels C and D: Analysis of the levels of active MMP-2 (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM. (a, P<0.001; b, P<0.05 vs treatment with GnRH I alone) in the bar graphs.
A.

Target/internal standard cDNA

GnRH II  0  100 nM  100 nM
Cetrorelix  0  0  100 nM

B.

Target/internal standard cDNA

GnRH II  0  100 nM  100 nM
Cetrorelix  0  0  100 nM
Figure 38: Effects of Cetrorelix on MMP-2 or MMP-9 mRNA and protein expression levels in EVT cultures in the presence of GnRH II. Panels A and B: QC-PCR analysis of MMP-2 or MMP-9 mRNA levels in untreated EVTs (lane 1), or cells cultured in the presence of a fixed amount of GnRH II (100 nM) alone or in combination with Cetrorelix (100 nM) (lanes 2 and 3, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below. Panels C and D: Analysis of the levels of active MMP-2 (Panel C) or MMP-9 (Panel D) present in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM in the bar graphs.
A. Target/internal standard cDNA

![Image A]

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B. Target/internal standard cDNA

![Image B]

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Figure 39: Effects of Cetrorelix on TIMP-1 mRNA and protein levels in EVTs cultured in the presence of GnRH I or GnRH II. Panels A and B: QC-PCR analysis of PAI-1 mRNA levels in untreated EVTs (lane 1) or cells cultured in the presence of a fixed amount of GnRH I or GnRH II (100 nM) and the GnRH antagonist, Cetrorelix, (lanes 2 and 3, respectively). The sizes of the resultant target and internal standard PCR products relative to a 100bp ladder (lane MW) are marked to the right of the photomicrograph. The absorbance values obtained from 5 independent studies are represented (mean ± SEM) in the bar graphs below (a, P<0.001 vs. treatment with GnRH I or GnRH II alone). Panels C and D: Analysis of TIMP-1 levels in the conditioned medium of these EVT cultures by ELISA. Data are shown as the means of five independent assays ± SEM (b, P<0.05 vs treatment with GnRH I or GnRH II alone) in the bar graphs.
6.6 DISCUSSION

Here we report that GnRH I and GnRH II are capable of increasing MMP-2 and MMP-9 and concomitantly decreasing TIMP-1 mRNA and protein levels in EVTs propagated from explants of first trimester chorionic villi. The regulatory effects of GnRH I, but not GnRH II, on MMP-2, MMP-9 and TIMP-1 expression in these cells was inhibited by Cetrorelix, a GnRHR specific antagonist (Neill 2002), suggesting that the biological actions of these two hormones on EVTs may be mediated by different receptor-mediated intracellular signaling pathways.

GnRH I expression in the human placenta is highest during early gestation (Pragar et al., 1992, Siler-Khodr et al., 2001), which correlates with maximum trophoblast invasion into the decidua (Pijnenborg et al., 1980, 1983, Aplin et al., 1991). Significant levels of GnRH II have also been detected in human placental tissues at all stages of gestation (Siler-Khodr et al., 2001). In addition to regulating MMP-2 and MMP-9 expression in EVTs, we have recently determined that both GnRH I and GnRH II increase the expression of urokinase plasminogen activator (uPA), another protease active at the maternal fetal interface (Chou et al., 2003). GnRH I and GnRH II may also promote the proteolytic activity of EVTs in vitro by virtue of their ability to coordinately decrease the expression levels of the endogenous inhibitors of these two families of proteases, TIMP-1 and plasminogen activator inhibitor (PAI)-1 respectively, in these cell
cultures (Chou et al., 2003). Collectively, these observations suggest that GnRH I and GnRH II play key roles in promoting the invasive capacity of human trophoblasts.

GnRH I and GnRH II were both capable of increasing the mRNA and protein levels of MMP-2 and MMP-9 in EVTs. To date, the mechanisms underlying these two distinct regulatory effects have not been determined. However, the increase in MMP-2 and MMP-9 mRNA suggests that one level of regulation may be elicited by an increase in gene transcription and/or increased mRNA stability (Nuttall et al., 2000). Similarly, increased MMP-2 and MMP-9 activity in these cell cultures may be owing to an increase in the production of the latent enzyme and/or decreased degradation of these enzymes (Nuttall et al., 2000). Our previous observations which indicate that uPA is coordinately regulated with MMP-2 and MMP-9 in EVTs in response to GnRH I or GnRH II suggests that the functions of these enzymes may be linked, possibly by participation as components of a proteolytic cascade. In particular, increased uPA expression in these cell cultures may lead to the generation of plasmin from plasminogen, which in turn, activates the latent form of MMP-9 (Kleiner et al., 1993). In addition, MMP-2 has been shown to activate MMP-9 activity in human cancer cells (Fridman et al., 1995).

GnRH II has been shown to mimic the biological actions of GnRH I in extrapituitary tissues including the placenta, ovary and endometrium. In the placenta, GnRH I and GnRH II
have been shown to stimulate the secretion of the beta subunit of human chorionic gonadotropin (βhCG) at the lowest concentration studied (Siler-Khodr et al., 2001). The stimulatory actions of GnRH II on βhCG secretion from placental tissue explants were greater than those observed with equal concentrations of native GnRH I or its synthetic analogs (Siler-Khodr et al., 2001).

In addition to the regulating the hormonal capacity of the human placenta, our studies suggest that both GnRH I and GnRH II may play key roles in modulating the invasive phenotype of human trophoblasts by virtue of their ability to regulate the balance between MMP and TIMP expression in these cells. However, GnRH II is capable of eliciting these regulatory effects at lower hormone concentrations than GnRH I. Similarly, GnRH II has been shown to be a more potent regulator of the steroidogenic capacity of granulosa-lutein cells (Kang et al., 2001) and the proliferation of human endometrial and ovarian cancer cells in vitro (Grundker et al., 2002).

These observations have led to the proposal that GnRH II may be the biologically active form of this hormone in extrapituitary tissues (Siler-Khodr et al., 2001).

Prolonged exposure to GnRH I resulted in a decrease in MMP-2 and MMP-9 mRNA and protein levels in the primary cultures of EVTs. Similarly, the inhibitory effects of GnRH I and GnRH II on TIMP-1 mRNA and protein levels in these primary cell cultures was also reduced with time in culture. This biphasic effect may be attributed to decrease in GnRHR-I expression levels, a biological phenomenon previously observed in the GnRH I-stimulated pituitary cells.
and villous cytotrophoblasts isolated from first trimester placental tissues (Cheng et al., 2000, Currie et al., 1993). In addition, GnRH I and GnRH II may subject to degradation by the high levels of C-ase-1 postproline peptidase detected in human placental tissues and primary cultures of human trophoblasts (Siler-Khodr et al., 1989, Kang et al., 1992).

GnRH II has been shown to bind the GnRH receptor (GnRHR) present in the human placenta with higher affinity than GnRH I suggesting that a receptor specific for GnRH II is present in this dynamic tissue (Siler-Khodr et al., 2001). The ability of Cetrorelix, to inhibit the regulatory effects of GnRH I, but not GnRH II, on MMP-2, MMP-9 and TIMP-1 mRNA and protein levels, provides further evidence that the biological actions of these two hormones are elicited by distinct receptors. Recently, a gene encoding a second receptor for GnRH (GnRHRII) has been identified in the human genome (Neill 2002, Millar et al., 2001, Morgan et al., 2003). Although a full-length mRNA transcript encoding this second form of human GnRHR has not been isolated, GnRHRII mRNA transcripts have been detected in total RNA extracts prepared from human term placenta (Neill 2002).

In summary, we have determined that GnRH I and GnRH II increase the mRNA and activity levels of MMP-2 and MMP-9 mRNA in primary cultures of EVTs propagated from first trimester placental tissues in a dose- and time-dependent manner. In contrast, these two hormones decreased the expression levels of the endogenous inhibitor, TIMP-1 in these cell
cultures. These findings strengthen our hypothesis that GnRH I and GnRH II play key regulatory roles in the proteolytic degradation of the extracellular matrix that occurs at the maternal-fetal interface during early pregnancy in the human.
PART VII. GENERAL DISCUSSION, SUMMARY, AND CONCLUSIONS

7.1: GENERAL DISCUSSION

The ECM at the maternal-fetal interface is subject to extensive remodeling, particularly during the early stages of pregnancy when the regulated invasion of trophoblasts into the maternal decidua and vasculature is paramount. These remodeling events are mediated by the regulated expression of uPA and members of the MMP gene family, particularly MMP-2 and MMP-9 in both the subpopulation(s) of invasive extravillous cytotrophoblasts and the deciduas, and counterbalanced by their respective endogenous inhibitors, PAI-1 and TIMP-1 in both the maternal and fetal compartments (Lala and Kearns, 1983, Bischof et al., 2000). Maximum activity levels these two proteolytic systems are observed during the first trimester of pregnancy and decline as the invasive capacity of trophoblasts is reduced with gestational age (Multhaupt et al., 1994; Polette et al., 1994; Shimonovitz et al., 1994). Similarly, GnRH I expression in the human placenta and endometrium is highest during early gestation (Pragar et al., 1997, Takeuchi et al., 1988) which coincides with maximum trophoblast invasion into the decidua. GnRH II expression is also regulated in human endometrium during the menstrual cycle and has been detected in first trimester and term human placental tissues (Siler-Khodr and Grayson, 2001). However, it is still unclear whether GnRH II is spatiotemporally expressed in these tissues during gestation. To date, the biological significance of GnRH I and GnRH II expression in these two dynamic tissues is not fully understood. However, in view of the direct correlation between the levels of GnRH I (and GnRH II) and the activity of the uPA and MMP proteolytic systems at the maternal-fetal interface, we hypothesized that GnRH I and/or GnRH II play key regulatory roles in the proteolytic degradation of the ECM of the endometrial stroma, a prerequisite for decidualization and the subsequent invasion of trophoblast. In these studies,
we have determined that GnRH I and GnRH II are capable of increasing the uPA and MMP expression levels in primary cultures of extravillous cytotrophoblasts and decidual stromal cells isolated from first trimester tissues in a dose- and time-dependent manner (Fig. 40). These observations strengthen our hypothesis that GnRH I and GnRH II are capable of regulating the proteolytic activity of decidual cells and the subpopulation(s) of trophoblasts present at the maternal interface in both an autocrine and paracrine manner.

GnRH II has been shown to mimic the biological actions of GnRH I in extrapituitary tissues (Leung et al., 2003). Furthermore, there is increasing evidence that GnRH II may be the biologically active form of this hormone in including the placenta, ovary and endometrium. For example, the antiproliferative effects of GnRH II on human endometrial and ovarian cancer cells were significantly greater than those observed in cells cultured in the presence of equivalent concentrations of native GnRH I or synthetic analogs (Grundker et al., 2002). GnRH II has also been shown to have a more potent inhibitory effect on the secretion of hCG by human term placenta and the steroidogenic capacity of granulosa-lutein cells in vitro (Kang et al., 2001; Sillier Khodr and Grayson, 2001). Similarly, we have determined that GnRH II is capable of eliciting regulatory effects on MMP-2, MMP-9 and uPA expression levels in human decidual stromal cells and extravillous cytotrophoblasts at lower hormone concentrations than GnRH I. In addition, we have demonstrated that GnRH I and GnRH II decrease PAI-1 expression in primary cultures of extravillous cytotrophoblasts but have differential effects on PAI-1 mRNA and protein levels in decidual stromal cells. To our knowledge, these studies are the first to demonstrate that GnRH I and GnRH II are capable of having differential biological actions on mammalian cells and suggest that the regulatory effects of these two hormones may be tissue/cell-specific.
The molecular mechanisms underlying the distinct regulatory effects of GnRH I and GnRH II on the MMP and uPA proteolytic systems in human extravillous cytotrophoblasts and decidual stromal cells has yet to be elucidated. The ability of Cetrorelix, a GnRHR-specific antagonist (Neill., 2002), to inhibit the biological actions of GnRH I but not GnRH II on these cells suggest that these effects may be elicited by differences in the binding affinity of GnRH I and GnRH II to one or more GnRH receptors and that there is minimal cross-reaction between the GnRH I- and GnRH II-specific receptors. Recently, a gene encoding a second receptor for GnRH (GnRHR-II) has been identified in the human genome (Millar et al., 2001; Neill, 2002). Although a full-length mRNA transcript encoding this second form of human GnRHR has not been isolated, GnRHR-II mRNA transcripts have been detected in the human endometrium and placenta (Neill, 2002). Alternatively, GnRH I and GnRH II binding to GnRHR may activate distinct intracellular signaling in human decidual stromal cells and extravillous cytotrophoblasts.

GnRH I binding to its receptor activates several intracellular signaling pathways including the PKA, PKC and/or MAPK cascades (Cheng and Leung, 2000). Recent studies indicate that the activation of ERK 1/2 signaling cascade by GnRH I involves the transactivation of the epidermal growth factor receptor (EGFR) (Shah et al., 2003). Interestingly, there is a marked increase in EGFR expression in the endometrial stroma as it undergoes decidualization, with maximum levels being detected in decidua cells present at the maternal-fetal interface (Lockwood et al., 2003). EGFR agonists have also been shown to increase PAI-1 expression levels in human endometrial stromal cells undergoing steroid-mediated decidualization in vitro (Lockwood et al., 2001; Lockwood, 2002). In contrast, invasive extravillous cytotrophoblast do not express significant levels of EGFR (Johki et al., 1994; Aboagye-Mathiesen et al., 1994). In view of these observations, it is tempting to speculate that the differential effects of GnRH on
PAI mRNA and protein expression levels in primary cultures of decidual stromal cells and extravillous cytotrophoblasts may be due to the presence or absence of EGR in these two cell types, respectively. In addition, GnRHR has also been shown to regulate LH and FSH gene expression via the PKC-mediated activation of the activator protein-1 (AP-1) (Strahl et al., 1998). Interestingly, AP-1 has been shown to be a key regulator of both MMP-9 and uPA gene expression in a wide variety of normal and malignant cells (Westermark et al., 1999; Vicenti et al., 2001) suggesting that GnRH I and/or GnRH II may have a direct stimulatory effect on the expression of these 2 genes in human extravillous cytotrophoblasts and decidual cells. To date, the intracellular signaling mechanisms activated by GnRH II remain poorly characterized.

The administration of Gonadotropin releasing hormone (GnRH) agonists and antagonists during early pregnancy has been shown to improve pregnancy rates in women undergoing IVF-ET (Casper, 1991, Wilshire et al., 1993; Weissman and Shoham, 1993) and in women diagnosed with unexplained, primary infertility (Smitz et al., 1991; Balasch et al., 1993). These beneficial effects have been attributed primarily to the direct actions of GnRH on the endometrium and/or placenta, although the underlying cellular mechanisms were previously unknown. Our studies support a direct effect of GnRH I and GnRH II on the human endometrium and placenta and indicate that the beneficial effects of GnRH on pregnancy outcome is mediated, at least in part via their ability to regulate the uPA and MMP proteolytic systems operative at the maternal-fetal interface during early pregnancy. Furthermore, our studies suggest that GnRH II should be targeted as drug for implantation-based infertility, other endometrial disorders, and endometrial-based contraception.
7.2 SUMMARY AND CONCLUSIONS

In summary, we have determined that GnRH I and GnRH II increase the uPA and MMP proteolytic systems in primary cultures of decidual stromal cells and extravillous cytotrophoblasts isolated from first trimester tissues. The biological effects of GnRH II in these primary cell cultures were consistently greater than those observed for GnRH I suggest that that GnRH II may be the biologically active form of this hormone, at least in these two reproductive tissues. In addition, we have demonstrated that GnRH I and GnRH II are capable of having differential biological actions on mammalian cells and suggest that the regulatory effects of these two hormones may be tissue/cell-specific. Collectively, these findings strengthen our hypothesis that the two forms of GnRH secreted by the placenta and endometrium play key regulatory roles in the ECM remodeling events that occur at the maternal-fetal interface during pregnancy in the human. The mechanisms by which GnRH I and GnRH II elicit their biological actions on the human endometrium and placenta warrants further investigation.
Decidual cell

Figure 40. Proposed effects and intracellular signaling pathways of GnRH I and II at maternal-fetal interface.
7.3 Future Studies

The results obtained in the present studies advanced our understanding the regulatory roles of GnRH I and GnRH II on proteolytic enzyme systems. However, the complete autocrine/paracrine functions of these two hormones in decidual and placenta tissue are still far from being understood. Further studies are certainly needed to improve our knowledge to unveil the mysteries of these hormones in decidual and placenta tissue. These include:

1) The localization of AP-1 and NF-κB cis-response element in the human MMP-9 5’-flanking region, suggests a potential regulation of this gene by both PKC and PKA pathways. Activation of these pathways in the decidual and placental cells by various hormones, including GnRH, provides a complex hormonal network to control the expression of this gene. Hence, the identification of signaling transduction pathways and further blocking the factors in these pathways by using RNAi techniques would certainly improve our understanding of the regulation of MMP-9 gene expression by GnRHs.

2) It is now known that the expression of uPA and MMP-9 is regulated by growth factors, receptor-type tyrosine kinases and cytoplasmic oncoproteins. We hypothesized that GnRHs activate uPA and MMP-9 collagenase promoters by Ets and AP-1 transcription factor binding sites. However, how these transcription factors form a complicate network in controlling the expression of the uPA gene has not been established. It will be interesting to identify the transcription factors that are involved in the expression of the uPA gene and to elucidate their role in controlling the uPA gene expression in human placenta or endometrium in response to GnRHs.
3) The differential role of GnRH I and GnRH II in mediating the PAI-1 effect in decidual cells was demonstrated in our previous studies. The differential effect of EGFR in these cells was suggested to account for this effect. It is therefore essential to elucidate the mechanism of this differential effect by these two hormones to disclose the relationship between GnRH and EGFR.

4) The mechanisms underlying the specificity of the transcriptional response to the activation of cell surface receptors by GnRH I and GnRH II are not well understood. Microarray techniques have emerged as important approaches for the simultaneous analysis of multiple gene transcripts. It is necessary to develop a cDNA microarray to study genes that are regulated by GnRH I and GnRH II in human placenta.
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