GONADOTROPIN-RELEASING HORMONE (GnRH) REGULATES TROPHOBLAST INVASION AND VASCULAR MIMICRY: INVOLVEMENT OF CELL-CELL ADHESION AND PROTEOLYSIS DYNAMICS.

by

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ABSTRACT

To date, the pro-invasive role of the GnRH-GnRHR system has been demonstrated in several cell types including carcinoma cells. Placental expression of GnRH I, GnRH II, and their mutual receptor (GnRHR) is indicative of a potential mechanism(s) that occurs during the dynamic process of human placenta formation and differentiation, particularly during the development of an invasive phenotype of extravillous trophoblasts (EVTs). However, current studies haven’t completely revealed the role of GnRH in regulating invasive EVT function and the underlying mechanism(s) is not yet well-established. Dynamic reprogramming of cell adhesion and proteolytic machinery is frequently accompanied with cell invasive and angiogenic phenotypes. I hypothesized that GnRH could regulate trophoblast invasion and vascular remodeling via modulation of mesenchymal cadherins and matrix matelloproteinases (MMPs). In these studies, I have found that both GnRH forms could regulate N-cadherin and cadherin-11 expression distinctly by activating of transcription factors TWIST and c-FOS/c-JUN, respectively. Furthermore, I have demonstrated that GnRH I and GnRH II are capable of increasing MMP-2 and MMP-9 expression in EVT cells via up-regulation of the transcription factor RUNX2. Specific inhibition of TWIST/N-cadherin, c-FOS/c-JUN/cadherin-11 and RUNX2/MMP-2/MMP-9 in EVT cells attenuates both basal and GnRH-induced trophoblast invasion. Additionally, both forms of GnRH stimulate matrigel-mediated
capillary-like network formation of trophoblastic cells and this phenomenon is also mediated by GnRH induced N-cadherin, cadherin-11, MMP-2 and MMP-9 expression. Collectively, our observations strengthen our hypothesis that GnRH is an important regulator of EVT cell behavior during implantation and placentation. These studies systemically described the underlying molecular mechanisms involved in GnRH induced adhesion molecule and proteolysis reprogramming.
PREFACE

My supervisor Dr. Peter C.K. Leung, my colleague Dr. Hua Zhu and I designed the experiments in Chapters 3, 4, 5, 6 and 7. Dr. Colin MacCalman assisted me in experimental designing in Chapters 4 and 5. I conducted all the experimental procedures in Chapters 3, 4, 5, 6 and 7 and Dr. Hua Zhu assisted me in immunohistochemistry staining of GnRHR, TWIST, N-cadherin and RUNX2 in first-trimester human placenta (Fig. 3. 5, Fig. 4. 1, A and Fig. 5.1, A).

All my studies in this thesis were conducted under the supervisory of Children’s and Women’s Research Ethics Board in the University of British Columbia. The project title is “Biological actions of GnRH in human implantation and placentation” and ethic file number is H07-01149.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium iron</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<tr>
<td>E₂</td>
<td>Estradiol</td>
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<tr>
<td>EC</td>
<td>Extracellular subdomain</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetate</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
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<td>Abbreviation</td>
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<tr>
<td>EVT</td>
<td>Extravillous cytotrophoblast</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>FGF receptor</td>
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<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
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<td>g</td>
<td>Gram</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
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<tr>
<td>HAV</td>
<td>Histidine-alanine-valine</td>
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<td>hCG</td>
<td>Human chorionic gonadotropin</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
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<tr>
<td>Kb</td>
<td>Kilo base</td>
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<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
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<tr>
<td>LH</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>PAGE</td>
<td>Polyacrylimide gel electrophoresis</td>
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<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
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<tr>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<td>SV40</td>
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<td>TGF-β1</td>
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<td>Tissue inhibitor of MMP</td>
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<tr>
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<td>Transmembrane domain</td>
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<td>Urokinase-type PA</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Accompanied with every step I go forward, there is always a tiny but strong step here by the side of me. This step belongs to a girl who always shares my laughter, who always wipes my tears, who always grants me confidence and who always tells me not to give up. I will dedicate this thesis to my wife Ms. Wei Wu to express my gratitude and love to her from the deepest bottom of my heart. No matter where I go on my way, no matter what I get in my life, I will always be the intrepid one just as long as you stand by me.
CHAPTER 1: OVERVIEW

1.1 Introduction

To maintain the basic survival and development of our human species, the process of human reproduction is extremely critical, although its efficiency is relatively low. At least 40% of human pregnancies cannot pass 20 weeks gestational age (Wilcox et al., 1988). Excluding fetal chromosomal and structural abnormalities, two important reasons for these early losses of pregnancy are abnormal implantation and inadequate placentation.

The human placenta plays critical roles not only in maintaining pregnancy but also in regulating fetal growth and development. The structure of the human placenta provides a physical link between the mother and growing fetus, and the vascular-abundant placental compartment efficiently transfers gas, nutrition, and waste across the maternal-fetal interface. Furthermore, the physical and immune barriers of the placenta also protect both mother and child from pathogens and immune responses, and the endocrine activities of the placenta provide growth factors, cytokines, and hormones, which are necessary for fetal development and pregnancy maintenance. Systemic reviews on placenta pathology shows that abnormal placental development is regarded as a major cause of several reproductive disorders, such as recurrent miscarriage, intrauterine growth restriction (IUGR), and preeclampsia (Norwitz, 2006, 2007). For example, superficial
placentation and subsequent reduced angiogenesis-related cytokines and growth factors have been reported to be related to preeclampsia (Steegers et al., 2010). Therefore, better knowledge of the specific cellular mechanisms involved in early implantation and placentation may benefit our understanding of these complicated processes.

The interaction between the blastocyst and uterus constitutes the initial step of a complicated process that leads to early implantation and placental development. After the initiation of the embryo-uterus interaction, the trophoblast, derived from the trophoectoderm layer of the blastocyst, contributes extensively to placenta construction on the fetal side. Trophoblastic cells undergo proliferation, differentiation, and invasion during their interaction with the maternal endometrium. Three populations of trophoblastic cells have been identified according to their structure and function: villous cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast (Hertig et al., 1956). The villous cytotrophoblast preserves its strong proliferative capability and is generally thought of as the “stem cell” of the entire trophoblast cell population. The syncytiotrophoblast is formed by cell fusion and forms the major maternal-fetal barrier for nutrition and oxygen transportation. The extravillous trophoblast (EVT), a highly differentiated and invasive cell population, provides strong physical anchorage between the embryo and the uterus and is also capable of vigorously remodeling the endometrial spiral artery (Red-Horse et al., 2004). Shallow trophoblast invasion and restricted endovascular spiral artery remodeling are generally implicated as critical causes of preeclampsia (Brosens, 1977; Guzin et al., 2005; Meekins et al., 1994). Therefore, studies
regarding the mechanism(s) of trophoblast invasion and angiogenesis may broaden our understanding of the physiology of human pregnancy and the pathology of certain implantation-related diseases such as preeclampsia. In this chapter, I will introduce human implantation and placentation, trophoblast differentiation, trophoblast invasion and angiogenesis, and the research model used in my study.

Many growth factors, cytokines, and hormones have been detected at the maternal-fetal interface, and their functions in regulating trophoblast invasion have been gradually revealed (Bischof et al., 2000; Lala and Chakraborty, 2003). As the central regulator of the reproductive system, GnRH has been detected in the placenta (Khodr and Siler-Khodr, 1978b; Siler-Khodr and Khodr, 1978). The mRNA and protein expression of GnRH receptor (GnRHR) has also been reported in human placenta by our group and others (Cheng and Leung, 2000; Lin et al., 1995; Wolfahrt et al., 1998), whereas the cellular localization of GnRHR in the human placenta, especially the first-trimester placenta, has yet to be demonstrated. Therefore, in Chapter 3, I will describe the identification of the expression and cellular localization of GnRHR in the first-trimester human placenta and trophoblastic cell models.

The function of GnRH in the placenta has been demonstrated on both the maternal and fetal sides. For example, on the maternal side, GnRH was reported to reduce proliferation and induce apoptosis in endometrial stromal cells (Huang et al., 2013; Taniguchi et al., 2013). On the fetal side, GnRH has been reported to regulate trophoblast invasion through different mechanisms by our research group (Chou et al., 2003c; Liu et
al., 2009). However, a comprehensive understanding of the molecular mechanisms involved in this complicated signaling network remains to be determined. Therefore, in Chapters 4, 5, 6 and 7, I will discuss several novel molecular mechanisms of GnRH in regulating trophoblast invasion and spiral artery remodeling behavior using an in vitro model.

Mesenchymal cadherins (e.g., N-cadherin, R-cadherin, and cadherin-11) have been previously reported to regulate cell motility in both early embryonic development and cancer metastasis. A previous paper from our group discussed the potential role of N-cadherin in regulating trophoblast invasion behavior (Ng et al., 2012). Abundant expression of cadherin-11 has also been demonstrated in first-trimester human placenta, especially in syncytiotrophoblasts and extravillous trophoblasts (MacCalman et al., 1996). In Chapter 4, I will discuss the role of GnRH in regulating N-cadherin expression and trophoblastic cell invasion. The regulatory role of GnRH in stimulating cadherin-11 expression will be discussed in Chapter 6.

Cell invasive behavior requires dynamic extracellular matrix (ECM) degradation in both physiological and pathological conditions. ECM remodeling occurs via the coordinated activities of various proteinases, including matrix metalloproteinases (MMPs). GnRH has been previously shown to induce the expression of MMP-2 and MMP-9 in trophoblastic cells (Chou et al., 2003c); however, the molecular determinants of such effects have yet to be elucidated. In Chapter 5, I will discuss the role of an important transcription factor, RUNX2, in mediating GnRH-induced MMP-2 and MMP-9
expression in the human first-trimester trophoblasts.

Another critical duty that the invasive trophoblastic cell must undertake is endometrial spiral artery remodeling. To date, only one research article has reported increased proangiogenic chemokines following GnRH administration in human trophoblasts (Cavanagh et al., 2009). However, the pro-angiogenic effect of GnRH using any angiogenic assay has not yet been validated. In Chapter 7, I will describe the pro-angiogenic function of GnRH and will demonstrate the important role of mesenchymal cadherin and MMP in regulating trophoblastic angiogenic behavior.

1.2 Implantation and placentation in human

1.2.1 Implantation

The implantation process in humans is poorly understood. After ovulation, the ovum is fertilized in the ampulla near the ampullary–isthmic junction, where it resides for approximately 72 hours (Croxatto, 2002). During this time, embryonic cells divide and compact to form a cell mass called a morula. Under the influence of ovarian steroids such as progesterone and the developing embryo itself, distinct gene expression profile appears in morula as it passes through the isthmus into the uterus (Cui et al., 2007). After the morula enters the uterine cavity, cell polarity is established and lineage differentiation
occurs following formation of the blastocyst (Rossant, 2004). The blastocyst "hatches" from the zona pellucida about 72 hours after its entry into the uterine cavity. A tiny hole is formed in the anembryonic pole of the zona pellucida from which the blastocyst escapes (Seshagiri et al., 2009). Biochemical communication between blastocyst and endometrium that occurs before, during, and after hatching prepares the initial signaling for implantation. For example, human chorionic gonadotropin (hCG) released from the blastocyst is essential for implantation, and is required for effective interaction between the blastocyst and the endometrial epithelium (Cha et al., 2012). Implantation begins 6 to 7 days after fertilization during a narrow timeframe referred to as the “implantation window” (Sharma and Kumar, 2012). Implantation can be divided into three stages: apposition, adhesion, and invasion (Aplin and Kimber, 2004). During the apposition stage, the embryonic pole of the blastocyst is oriented towards the endometrium. Under the influence of progesterone, the endometrium forms numerous epithelial membrane projections called pinopodes. Pinopodes directly interact with microvilli on the surface of the trophoectoderm, and mediate adhesion via molecules such as E-cadherin present (Quinn and Casper, 2009). The location of apposition and adhesion is determined by the spatial expression of E-cadherin and other adhesion molecules such as integrins, laminin, fibronectin, and MUC-I in the maternal-fetal interface under the influence of endometrium- and blastocyst-derived cytokines (Aplin et al., 2000a; Giudice, 1999). After completion of blastocyst and endometrium adhesion, trophoblasts will penetrate the uterine epithelium and invade into the endometrium.
Many transcription factors tightly control the dynamic molecular changes during trophoectoderm formation and early trophoblast differentiation. For example, caudal-related homobox protein Cdx2 is one of many transcription factors that are highly expressed in the trophoectoderm layer (Strumpf et al., 2005). Overexpression of Cdx2 gene in mouse embryonic stem (ES) cells induces trophoblast formation (Red-Horse et al., 2004). Moreover, many hormones and growth factors also play crucial roles in implantation and early trophoblast lineage commitment. For example, in mice, external administration of estrogen could induce the window of uterine receptivity (Ma et al., 2003). Interestingly, knock-out studies in mice have demonstrated that uterine expression of estrogen receptor (ER) α and progesterone receptor (PR) A is essential for implantation whereas ER β and PR B are not required (Conneely et al., 2002; Hewitt and Korach, 2002). Bone morphogenic protein (BMP) 4 has also been shown to promote ES cell differentiation towards trophoblastic cell-types. As a tightly regulated and highly coordinated developmental process, implantation requires orderly regulation by multiple factors as well as in multiple levels.

1.2.2 Trophoblast differentiation

After the initiation of blastocyst-endometrium interaction, at day 6-8 after fertilization, the expanding trophoectoderm population starts to differentiate into two
layers, a thin layer of proliferative stem-cell like cytotrophoblast and an expanding pool of multinucleate syncytiotrophoblast via cytotrophoblast fusion (Hertig et al., 1956). Evidence to date suggests that both cytotrophoblasts and sycytiotrophoblast are involved in the initial invasion process, as both of them directly interact with the endometrial epithelial cells (Enders, 1976). The rapid expansion of the syncytiotrophoblast replaces the existing endometrial connective tissue, and is accompanied by stromal cell programmed cell death. At day 9-10 after fertilization, internal spaces called lacunae begin to appear within the continuously growing syncytiotrophoblast compartment. Endometrial blood sinuses interact with syncytiotrophoblast and maternal blood infiltrates into lacunae at day 11-12 after fertilization. Expansion of the lacunae isolates the continuous syncytiotrophoblast into a cord-like structure, penetrated by cytotrophoblastic cells which form the inner layer of primary placenta villi.

Surrounded by the syncytiotrophoblast layer, rapidly proliferating cytotrophoblastic cells begin to form the peripheral cell population of placenta villi. Extra-embryonic mesoblasts also migrate between cytotrophoblastic cells, and form the inner core of placenta villi. These mesoblasts will differentiate into mesenchymal cells and undifferentiated stromal cells, and could still be found until week 7-8 after pregnancy.

The growing embryo will eventually leave the endometrial bedding and elevate into the uterine cavity, accompanied by active placental villi elongation. A portion of the cytotrophoblastic cell population will directly interact with the endometrial decidua and form a cell cluster called a cell column. Cytotrophoblastic cells adjacent to villous
stromal cells maintain a high proliferative capacity and are called proximal column cytотrophoblasts. A subset of cytотrophoblastic cells gradually leaves the cell replication cycle, disseminates towards the endometrium and become EVTs by adopting an invasive phenotype.

Overall, in first-trimester human placenta, proliferative villous cytотrophoblasts, syncytiотrophoblast and EVTs are the three major trophoblast cell populations appear at the maternal-fetal interface (Fig 1.1).
Figure 1. A schematic diagram of the distribution of trophoblastic cell populations in first-trimester human placental villi.
1.2.3 Villous cytotrophoblast

Villous cytotrophoblasts are generally considered as progenitor cells of all the trophoblast cell populations, including syncytiotrophoblast and EVTs. Continuous proliferation of villous cytotrophoblasts is key to supporting the rapid growth of the placenta during pregnancy (Redman and Sargent, 2007).

The cell proliferation marker Ki67 and cell cycle regulator cyclin A are detected in villous cytotrophoblasts (Korgun et al., 2006). Rapid DNA synthesis has also been observed in villous cytrophoblastic cells, with estimates of mitotic index approaching 1.5-2.9% at 6-9 weeks of pregnancy. In contrast, no mitotic behavior has been observed in syncytiotrophoblasts (Chan et al., 1999; Kar et al., 2007).

Asymmetric cell division is a unique character of villous cytotrophoblasts throughout pregnancy. Some daughter cells of villous cytotrophoblasts must maintain their proliferative capability and serve as sustainable progenitor pool, and other daughter cells will differentiate towards intermediate cell type before fusion (Mayhew, 2001). Unbalanced signaling, polarized cell fate determination and uneven mitotic spindle assembly are required for the asymmetric cell division phenomenon (Gonczy, 2008).

Several molecular markers like cytokeratin-7 (Muhlhauser et al., 1995), cell adhesion molecule E-cadherin (Floridon et al., 2000; Li et al., 2003) and transcription factor GCM-1 (Baczyk et al., 2004) are abundantly expressed by villous cytotrophoblasts.
The presence of these cell markers indicates the epithelial origin of villous cytotrophoblasts. “Intermediate” villous cytotrophoblasts that are preparing for fusion have also been identified by transmission electron microscopy (Boyd and Hamilton, 1966; Jones and Fox, 1991). Moreover, differential expression of hCG α subunit (Hoshina et al., 1982) and fibroblast growth factor (FGF) receptor 2 (Baczyk et al., 2006) between cytotrophoblasts also provides evidence that some villous cytotrophoblasts have begun to differentiate towards a syncytiotrophoblast cell-type.

As for hormone secretion, villous trophoblasts play an important role in synthesizing activin, inhibin (Petraglia et al., 1996; Petraglia et al., 1991), and GnRH (Khodr and Siler-Khodr, 1980; Miyake et al., 1982). Many pro-angiogenic growth factors like vascular endothelial growth factor (VEGF), placenta growth factor (PIGF) and angiogenin are also expressed in villous cytotrophoblastic cell population (Lash et al., 2010).

1.2.4 Syncytiotrophoblast

Triggered by different internal and external influences, a subset of villous cytotrophoblastic cells differentiate along two distinct pathways by separated cell fate determination. At the surface of placental villi, cytotrophoblasts undergo fusion and differentiation to become syncytiotrophoblast. At the placenta-decidual contact region,
cytotrophoblasts differentiate along the invasive pathway to become EVTs.

Mononucleate cytotrophoblasts form multinucleate syncytiotrophoblast by cell fusion. Generally recognized as a syncytium, the syncytiotrophoblastic layer contains millions of nuclei and massive connected cellular membrane, covering up 12-14 m² of surface area at term (Burton and Jauniaux, 1995). Syncytiotrophoblast does not possess a lateral cell membrane. Polarized apical and basal cellular membranes are the only boundaries that isolate syncytiotrophoblast from maternal blood or villous cytotrophoblasts. The apical membrane of this syncytium contains microvilli, which expands the membrane surface size by five to seven fold (Karimu and Burton, 1995; Teasdale and Jean-Jacques, 1985). The basal membrane of the syncytium interacts directly with the villous cytotrophoblastic cell membranes during the first-trimester. After the rapid placental growth during the second and third trimesters, the syncytium becomes the only continuous barrier between maternal blood and fetal placental stromal cells. Thereafter, the syncytiotrophoblastic layer assumes the critical role of preventing the infiltration of toxins, pathogens and maternal cells. A layer of glycocalyx covers the syncytial microvilli which play important roles in preventing thrombosis and immunoreactions. Moreover, class I or II human leucocyte antigens (HLAs) are not expressed at the apical surface of the syncytiotrophoblast, which may also serve as immune tolerance regulators (Moffett and Loke, 2006).

The syncytiotrophoblast constitutes the outmost layer of the human placenta villi and directly contacts the maternal blood supply. Oxygen and nutrition need to cross the
continuous apical and basal syncytiotrophoblast cellular membrane from maternal side to fetal side. High densities of transporter proteins are expressed at both the apical and basal syncytial membranes (Cleal and Lewis, 2008; Sibley, 2009). Oxygen crosses the placenta primarily via unfacilitated diffusion, whereas glucose transport involves facilitated diffusion. Several members of the glucose transporter family (e.g. GLUT1, GLUT 3, GLUT 4 and GLUT 12) are expressed in syncytial membrane (Ericsson et al., 2005; Gude et al., 2003; Jansson et al., 1993), and play coordinate roles in glucose transportation. Amino acids require active transport to cross the placenta (Lewis et al., 2013). Taken together, the syncytiotrophoblast plays an important role in regulating the exchange of oxygen, nutrients and waste across the maternal-fetal interface.

The syncytiotrophoblast has bigger nuclei, larger number of mitochondria, well-developed rough endoplasmic reticulum (RER), and complicated Golgi apparatus, all of which provides essential machinery for rapid protein synthesis. The syncytiotrophoblast synthesizes large quantities of hormones, such as hCG and human placental lactogen (hPL) (Boime et al., 1982). Moreover, syncytiotrophoblast can also secret progesterone and leptin, contributing largely to pregnancy maintenance and metabolic regulation (Henson and Castracane, 2000; Paul et al., 1981).

Various signaling pathways, hormones and membrane proteins are known to regulate cytotrophoblast fusion (Huppertz and Gauster, 2011). For example, treatment with cyclic adenosine monophosphate (cAMP) analogue promotes cytotrophoblast fusion, which is inhibited by the cAMP dependent protein kinase inhibitor H-89 (Keryer et al.,
The placenta-specific envelop protein syncytin-1, encoded by human endogenous retroviral (Bai et al.) genes, is abundant in syncytiotrophoblast. Overexpression of syncytin-1 induces human choriocarcinoma BeWo cell fusion in vitro (Mi et al., 2000). Similarly, a second villous envelop protein, syncytin-2, has also been reported to induce cell fusion. Moreover, the $\text{Ca}^{2+}$ dependent cell adhesion molecule Cadherin-11 is highly expressed in syncytiotrophoblast and is also associated with cell fusion (Getsios and MacCalman, 2003; MacCalman et al., 1996).

### 1.2.5 Extravillous trophoblast (EVT)

In contrast to villous cytotrophoblasts and syncytiotrophoblast, extravillous trophoblasts are located outside the placental villi. All EVT cells are derived from the trophoblastic cell column, where placental villi directly interact with maternal decidua. At the anchoring site, the heterogeneous trophoblast population is not only capable of sustaining the cell column, but also serves as the cellular source from which EVTs differentiate (Vicovac et al., 1995).

Differentiation of cytotrophoblastic cells along the invasion pathways requires tissue structural reconstitution and cell morphological changes. The first cell layer that is adjacent to the basement membrane (basal lamina) of the cell column displays pure epithelial characteristics, and is the progenitor of the entire EVT population.
Differentiation is initiated in the first few cell layers which comprise the proximal region of the cell column. At this stage the cells maintain a relatively high proliferative capability but begin to acquire a migratory phenotype. In the distal region of the cell column, proliferative capacity gradually declines as daughter cells move away and begin to invade into the endometrium.

During the first- and second-trimesters, EVTs that are close to the basal lamina are highly proliferative and express proliferation markers such as Ki67, MIB-1 and proliferating cell nuclear antigen (PCNA) (Korgun et al., 2006). These proliferation markers have their specific expression patterns in cell column and EVTs in vivo. For example, DNA synthesis indicator $^3$H-thymidine could only label the first one or two layers of cells near the basal lamina, and Ki67/MIB-1 could be detected in the proximal region of cell column. In contrast, expression of PCNA is not only limited at cell column region, but also is expressed at invaded EVTs (King and Blankenship, 1994).

After the EVT cells leave the distal end of cell column, they undergo a morphological and functional change to spindle-like, polarized and highly invasive cells called interstitial EVTs. Interestingly, some interstitial EVT cells display a distinct polygonal shape and larger size, suggesting that further differentiation may occur during interstitial EVT invasion into the endometrium and myometrium. In humans, interstitial EVTs can be found as deep as one third of the myometrium, where huge multinucleated giant cells are formed. The major function of interstitial EVTs is to physically anchor the placenta to the endometrium; however, a role in immune regulation is also likely since
they interact extensively with maternal immune cells during the invasive process (Oreshkova et al., 2012).

Some EVT cells can be found inside the endometrial spiral arteries, and are referred to as endovascular EVTs. These cells move into the vascular endothelium of maternal spiral arteries either by migrating through the endometrium towards a vascular opening, or by directly penetrating the vascular wall from outside the spiral artery. Endovascular EVTs replace maternal vascular endothelial cells and remodel the morphology of spiral arteries from low dilation to high dilation. The major purpose of trophoblasts’ spiral artery remodeling behavior is to ensure sufficient blood flow and to provide accelerated material exchange, further satisfying the increasing nutrition and oxygenation demands of the rapidly growing fetus.

EVT differentiation along the invasive pathway is associated with significant changes in the expression of cell markers, cell adhesion molecules, extracellular matrix components and proteolytic enzymes. The presence of cytokeratin in the whole EVT cell population shows its epithelial origin. In contrast to villous cytotrophoblasts, EVT cells uniquely express class I human leukocyte antigen (HLA) HLA-G (Loke et al., 1997; Sasagawa et al., 1987). Differentiation from villous cytotrophoblastic cells to EVTs has also been suggested to involve a loss of EGFR and a gain of HER2 oncoprotein (Jokhi et al., 1994).

During cell invasion, EVT cells interact with distinct ECM components which differ from those of the villous epithelial environment. Villous cytotrophoblasts secrete collagen
IV as their major ECM component, whereas fibronectin is the predominant ECM component in the cell column. Laminin becomes the dominant ECM component as EVTs invade more deeply into the endometrium (Benirschke et al., 2012).

A dynamic integrin switch is often associated with ECM change during EVT invasion. Integrin α6β4 is predominantly expressed in the basal lamina of placental villi. Downregulation of α6β4 and upregulation of α5β1, α4β1 has also been found during EVT differentiation inside the cell column. Integrin β1 is maintained at high levels during EVT invasion, however α5 and α4 are gradually replaced by α1 and α6 as EVTs invade deeper into the endometrium (Damsky et al., 1992).

As the primary component of adherens junctions, cadherins also display unique patterns of expression in EVTs compare to villous cytotrophoblasts. Loss of epithelial E-cadherin and gain of mesenchymal cadherin-11 in EVTs is associated with the acquisition of invasiveness (Damsky and Fisher, 1998; MacCalman et al., 1996). Interstitial and endovascular EVTs have also been shown to express the endothelial marker VE-cadherin, which is thought to contribute to trophoblastic endovascular differentiation or “vascular mimicry” (Damsky and Fisher, 1998).

Acquisition of the capacity for ECM degradation/remodeling is an important characteristic of EVT cells. Secretion of MMPs, primary gelatinases MMP-2 and MMP-9, and urokinase-type plasminogen activator (uPA) has been reported in EVT cells. Notably, tissue inhibitor of metalloproteinase-1 (TIMP-1) and plasminogen activator inhibitor 1/2 (PAI-1/2) are also detected in EVT cells, indicating local regulation of the proteolytic
capabilities of EVTs which is critical to preventing excessive EVT invasion (Hofmann et al., 1994; Huppertz et al., 1998).

1.2.6 Endocrine factors regulating EVT invasion and spiral artery remodeling

Invasion and spiral artery remodeling behaviors of EVT cells are tightly regulated by a variety of growth factors, cytokines and hormones, each operating through distinct mechanisms. For example, insulin-like growth factors (IGF) are detected in both villous mesenchymal cells and cytotrophoblasts, with an increased expression at the distal end of cell column (Han et al., 1996). Conditioned medium containing IGF increases trophoblast cell invasion in vitro, whereas removing IGF from the cultures reduces cell invasiveness (Aplin et al., 2000b). Other growth factors like EGF, heparin-binding EGF-like growth factor (HB-EGF), FGF, colony stimulating factor and hepatocyte growth factor have all been reported to promote EVT invasion (Knofler, 2010). As well as increasing invasiveness, growth factors are also important negative regulators of EVT invasion. For example, transforming growth factor β (TGFβ) restrains trophoblast invasion in vitro by attenuating VE-cadherin expression and MMP-9/uPA secretion (Lash et al., 2005). Leukemia inhibitory factor (LIF) and tumor necrosis factor α (TNFα) are also known to inhibit trophoblast invasion by regulating trophoblast-ECM interactions (Hunt, 1989; Tapia et al., 2008).
Cytokines secreted by uterine natural killer cells also regulate trophoblast invasion, indicating trophoblast behavior is tightly controlled by maternal signals. For example, uterine natural killer cell-derived interleukin-8 (IL-8) stimulates EVT invasion in explants of first-trimester placental villi (De Oliveira et al., 2010). In contrast, IL-12 is thought to suppress MMP expression and activate TIMPs, thereby inhibiting trophoblast invasiveness (Karmakar et al., 2004).

Hormones such as hyperglycosolated-hCG (Cole, 2007, 2010; Handschuh et al., 2007), leptin (Castellucci et al., 2000) and GnRH (Chou et al., 2003c) have also been shown to stimulate trophoblast invasion by regulating the expression of MMPs.

The process of uterine spiral artery remodeling during placental development shares a number of features with the angiogenesis process, either physiologically or pathologically (e.g. tumor angiogenesis). Several angiogenic factors, such as VEGF, PlGF and angiopoietin 2, are expressed by EVTs (Zhou et al., 2003; Zhou et al., 2002). These angiogenic factors stimulate chicken chorioallantoic blood vessel growth in vitro, and removal of these factors leads to massive cell apoptosis in cultured trophoblasts (Zhou et al., 2003).
1.2.7 Shallow EVT function and preeclampsia

Preeclampsia is a pregnancy specific disease which is characterized by *de novo* maternal hypertension and proteinuria. Severe preeclampsia could lead to eclampsia, which is the major cause of maternal death during pregnancy. The etiology of preeclampsia is complicated and not fully understood, however shallow trophoblast invasion and inadequate spiral artery remodeling are generally believed to be important contributors to the development of preeclampsia. Several studies have linked superficial trophoblast invasion and restrained trophoblast infiltration into spiral artery to preeclampsia (Brosens et al., 1972; De Wolf et al., 1975). In particular, the failure to switch from “low-diameter high resistance” spiral arteries to “high-diameter low resistance” vessels is thought to be one vital causes that leads to preeclampsia (Burton et al., 2009). Upregulated soluble fms-like tyrosine kinase-1 (sFlt-1), a soluble VEGF receptor, has been reported in preeclamptic patients (Maynard et al., 2003). Elevated maternal plasma sFlt-1 levels have been shown to precede the onset of preeclampsia (McKeeman et al., 2004), and sFlt-1 levels are correlated to disease severity (Levine et al., 2004). Therefore, a better understanding of the roles that invasion and angiogenesis related molecules play in early pregnancy has the potential to provide important insight into the mechanism(s) of pregnancy related disease such as preeclampsia.
1.3 Models in placenta research

1.3.1 Animal model

1.3.1.1 Mouse

Due to their small size and shorter reproductive cycle, mice have been widely used in implantation and placentation studies. The development of transgenic mouse models has significantly increased our understanding of the roles of specific genes during placental development. Many of the genes that play critical roles in human placental development are also important in mouse placentation (Rossant and Cross, 2001). Comparative homologue and structure similarity between human and mouse have been studied for many years (Georgiades et al., 2002). At maternal-fetal interface, the human placental villi tree structure and mouse labyrinth share many similarities; however, there are still a number of important differences between human and mouse placentation. For example, trophoblast invasion in mice is shallow (Pijnenborg et al., 2006), and they lack a comparative cell-type to the highly invasive interstitial EVT cells seen in humans. Moreover, mouse natural killer cells have more a profound function in vascular remodeling compared to those in human (Monk et al., 2005). These limitations may limit the use of mouse models in human placentation research.
1.3.1.2 Other mammals

Sheep have a longer development time *in utero* compared to mice, and fetal lambs have a birth weight similar to that of humans. In studies related to placental oxygen transportation, sheep are widely used as they have been established as a superior model compared to other mammalian species (Richardson and Bocking, 1998). However, the placenta of sheep is epithelialchorial in nature, which means that maternal blood does not directly interact with fetal tissue. Moreover, sheep have a relatively non-invasive placenta characterized by a dearth of trophoblast invasion. Owing to these disadvantages, sheep are not generally considered to be a suitable model for the study of trophoblast invasion and spiral artery remodeling.

Lemurs, lorisoids, tarsiers, New and Old World monkeys, gibbons and the great apes are all non-human primates. The placenta of great apes is very similar to that of humans, and substantial interstitial invasion is found in all the great apes. However, non-human primates in placenta research are limited due to their lack of availability, expensive price and increasing ethical concerns.

Due to numerous of limitations from animal models and accessibility of human placenta, many researches are conducted using tissues and cells from disposed human placenta.
1.3.2 Primary culture of human trophoblastic cells

Method for the isolation of human trophoblasts from chorionic villi using enzyme digestion was first reported by Kilman, et. al. and subsequently modified by several other groups (Douglas and King, 1989; Fisher et al., 1989; Kliman et al., 1986). This method has been widely used to isolate trophoblast cells from term placenta, and can also be used with samples of first trimester placental villi. Briefly, placental debris are incubated in trypsin or collagenase mixed with DNAase, and a Percoll gradient is used to separate trophoblast cells from other cell types such as red blood cells. Additional purification steps involving fluorescence-activated cell sorting (FACS) or affinity isolation with magnetic beads have also been described (Loke et al., 1989; Potgens et al., 2001). The epithelial cell marker cytokeratin 7 is widely used to distinguish trophoblastic cells from other cell types; however, it cannot discriminate between villous cytotrophoblasts and EVTs. Additionally, placental villous fibroblasts, decidual cells and stromal cells in culture could complicate cell purification.

The method specifically designed to isolate first trimester EVTs has been reported (Graham et al., 1993; Yagel et al., 1989). Briefly, first trimester placental villi are isolated and regions with abundant placental tips are collected. Placental villi are minced and placed in collagen coated or non-coated culture flasks, where cell columns attach and EVTs migrate outward from the cell column region. These cells have been shown to express EVT specific markers (e.g. HLA-G) and are generally considered to be EVTs.
However, placental villous fibroblasts also migrate out from the villi debris, therefore isolation of primary EVTs from fibroblast contamination is a challenge.

1.3.3 Trophoblastic cell lines

1.3.3.1 Choriocarcinoma cell lines

Limitations in the availability and use of first trimester human placenta samples has contributed to the extensive use of choriocarcinoma-derived cell lines in implantation and placentation research (King et al., 2000). Three choriocarcinomas, BeWo, JEG-3 and JAR are most widely used in trophoblast researches.

BeWo cells undergo spontaneous cell fusion and extensive cell fusion can be induced by treatment with forskolin or cAMP (Wice et al., 1990). This unique characteristic of BeWo cells makes them particularly well-suited for studies related to trophoblast fusion.

Treatment of JEG-3 cells with cAMP leads to elevated hCG-β production rather than cell fusion (Burnside et al., 1985; Chou et al., 1978; Coutifaris et al., 1991). Considered to represent mononucleate cytotrophoblasts, JEG-3 cells are relatively non-invasive and have been widely used in studies related to trophoblast differentiation along the invasive pathway. Isolated from a trophoblastic tumor in the placenta, JAR cells grow as spheroids
when placed on endometrial cells and are thought to exhibit some stem cell characteristics that make them useful as a model of trophoblasts with differentiation potential (White et al., 1988).

1.3.3.2 HTR-8/SVneo cell, an immortalized EVT cell line

Primary EVT cells have a limited life-span and can lose some of their characteristics during continuous culture. The immortalized EVT cell line HTR-8/SVneo was generated by transfection of parental HTR-8 cell with simian virus large T antigen. HTR-8/SVneo cells express cytokeratin, secret hCG and MMP-2, and exert high invasive capability (Graham et al., 1993). A gene profile analysis of comparing BeWo, JEG-3, JAR, HTR-8 and another immortalized EVT cell line SGHPL-5 with primary EVTs shows that immortalized EVT cells are different from primary EVT cells, although they still retain significant similarity and remain an important model of primary EVTs (Bilban et al., 2009).
1.4 GnRH and its receptor in placentation

1.4.1 GnRH and GnRHR

GnRH is a decapeptide which is secreted by hypothalamic neurons in a pulsatile manner. GnRH travels through the hypothalamo-hypophyseal portal system and targets gonadotropes in the anterior pituitary. GnRH binds to its G-protein coupled receptor GnRHR and stimulates follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion in gonadotropes. GnRH and its receptor have also been found in several extrapituitary tissues such as prostate, testis, mammary gland, ovary, endometrium and placenta (Chou et al., 2004; Fraser et al., 1974; Griesinger et al., 2006; Islami et al., 2001).

Several GnRH isoforms have been reported in human and other species (Fig.1.2, A). GnRH I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) was the first GnRH to be isolated and is mainly produced in the hypothalamus. The human GnRH I gene is located on chromosome 8p11.2–p21, has four exons and three introns, and encodes a precursor protein that contains 92 amino acids (Radovick et al., 1990). Originally isolated from chicken, a second GnRH (GnRH II, pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2) is conserved in most vertebrate species and also has been detected in human (Millar, 2003). In humans, the GnRH II gene is located on chromosome 20p13 and also contains four
exons and three introns. Compared to GnRH I, GnRH II is expressed at higher levels in human peripheral tissues such as prostate, kidney and bone marrow (White et al., 1998). Moreover, GnRH II has been reported to have significantly lower efficiency in gonadotropin stimulation compared to GnRH I (Densmore and Urbanski, 2003). These findings may indicate a more prominent role for GnRH I in the regulation of gonadotropin secretion, whereas the predominant functions of GnRH II are likely peripheral. Both GnRH I and GnRH II have been detected in first trimester human placenta on both the maternal side and the fetal side (Casan et al., 1998; Wolfahrt et al., 1998), whereas only GnRH I mRNA was detected at term placenta (Chou et al., 2004). Some research has also shown that both the GnRH isoforms are expressed in EVT and villous cytotrophoblast, whereas syncytiotrophoblast selectively expresses GnRH I (Chou et al., 2004).

GnRHR is a classical G-protein coupled receptor (GPCR) that contains seven transmembrane domains (Fig.1.2.B). It is primarily located in pituitary gonadotrope cells, but has also been found in many extrapituitary tissues and their associated malignancies. The GnRHR gene is located on chromosome 4q13.2–21.1 in humans, and it contains three exons that are separated by two introns. In contrast to other GPCRs, GnRHR displays resistance to receptor desensitization due to its lack of an intracellular cytoplasmic tail. GnRHR mRNA has been detected in human placenta (Cheng et al., 2000), but no study has shown the expression pattern of GnRHR protein. A second GnRH receptor GnRHR II has also been reported in several species, including non-human
primates (Millar et al., 2001). But later studies have failed to obtain full length GnRHR II transcript from human genome due to the presence of a frameshift and a premature stop codon (UGA) in the GnRHR II gene homologue (Morgan et al., 2003).

**Figure 1.** 2 Human GnRH isoforms (A) and signaling mechanisms (B).
1.4.2 GnRH in placentation and trophoblast invasion.

A well-established function of GnRH in the placenta is to regulate β-hCG secretion. β-hCG is secreted by trophoblastic cells in a pulsatile manner, and the level of β-hCG peaks in the first-trimester at 8-12 weeks of gestation. The critical presence of β-hCG in early pregnancy prevents the corpus luteum from degenerating, thereby ensuring adequate progesterone production. GnRH has been shown to stimulate hCG secretion in both primary syncytiotrophoblasts and choriocarcinoma BeWo cells (Butzow, 1982). Moreover, incubation with GnRH increases the frequency and amplitude of hCG secretion in trophoblast explants (Szilagyi et al., 1992). Interestingly, it appears that GnRH I stimulates hCG synthesis and secretion in trophoblast explants at a lower concentration, and for a longer period of time, compared to GnRH II (Islami et al., 2001).

Both GnRH I and GnRH II have been shown to induce primary extravillous trophoblast and immortalized cytotrophoblast cell invasion (Liu et al., 2010; Liu et al., 2009). In contrast to hCG production, GnRH II appears to be more potent than GnRH I in inducing trophoblast invasion (Liu et al., 2010). Several mechanisms have been described regarding the regulatory role of GnRH in trophoblast invasion. For example, GnRH regulates MMP-2, MMP-9, MMP-26 and TIMP-1 expression in EVT cells (Chou et al., 2003c; Liu et al., 2010; Liu et al., 2009). In addition, GnRH has been shown to upregulate uPA expression in both decidual cells and EVTs (Chou et al., 2003a). These
previous findings indicate that GnRH may serve as a local endocrine regulator in stimulating trophoblast invasion. However, the underlying molecular mechanism(s) remain to be fully elucidated.

1.5. Cadherins in trophoblast invasion

1.5.1 Adherens junctions and cadherins

An adherens junction is composed of the plasma membrane from two different cells with cadherin bridges in the intercellular space and actin filaments in the adjacent cytoplasmic regions (Miyaguchi, 2000). In polarized epithelia, the formation of an adherens junction at the apical regions results in a continuous “adhesion belt” between neighboring cells, with intercellular gaps of 10-20nm. In non-epithelial cell types such as fibroblasts, spot-like and aggregated adherens junctions are often observed (Yonemura et al., 1995).

Cadherins are a group of calcium-dependent proteins that are generally found in cell junction regions. The vertebrate cadherin superfamily contains more than 100 members which, based on structures and evolutionary relationships, and can be grouped into classic cadherins, protocadherins, desmosome cadherins, Flamingo/CELSRs (Cadherin EGF LAG seven-pass G-type receptor) and FAT-like cadherins (Fig. 1.2) (Redies et al., 2005;
Most cadherins are single-transmembrane proteins, however Flamingo/CELSRs have seven-transmembrane domains. Cadherins possess extracellular calcium binding domains called cadherin repeats or EC domains, which can vary from 1 to 34 repeats between different cadherins. Additional structures such as EGF-like domains, laminin globular-like domains and HormR domains can also be present in extracellular regions (Hulpiau and van Roy, 2009). The intracellular domain of cadherins is more diverse in composition among the various family members, and mediates interactions with various cytoplasmic proteins to trigger different signals (Oda and Takeichi, 2011). The major role of classic cadherins and desmosome cadherins is to assemble adherens junctions and desmosomes, respectively. Mediating cell-cell adhesion, regulating cell polarity and participating in cell differentiation are the primary functions of these cell-cell junctions. In contrast, other cadherin members such as protocadherins, Flamingo/CELSRs and FATs are less involved in mediating cell adhesion.

All the classic cadherins in vertebrates possess five extracellular cadherin repeats, as well as a relatively well-conserved intracellular domain which binds cytoplasmic p120-catenin and β-catenin (Tepass et al., 2000; Wheelock and Johnson, 2003). Another catenin member, α-catenin, is recruited to β-catenin and indirectly interacts with classic cadherins. The cadherin and catenin complex is linked to the cytoskeleton as a result of the binding affinity of α-catenin for actin filaments (Drees et al., 2005; Rimm et al., 1995). The binding of classic cadherins between adjacent cells is generally homophilic, however heterophilic interactions have also been described (Shimoyama et al., 2000).
Vertebrate classic cadherins contain more than 20 members which can be further subgrouped into type I and type II classic cadherins. E-cadherin (Epithelial-cadherin, CDH1), N-cadherin (Neural-cadherin, CDH2), P-cadherin (Placental-cadherin, CDH3), R-cadherin (Retinal-cadherin, CDH4) and M-cadherin (Myotubule-cadherin, CDH15) are all type I classic cadherins. All type I classic cadherins contain a highly conserved (His-Ala-Val) HAV motif in the first cadherin repeat (EC1), whereas type II classic cadherins, such as VE-cadherin (Vascular Endothelial-cadherin, CDH5) and Cadherin-11.
(OB-cadherin, Osteoblast-cadherin, CDH11), do not possess a HAV motif.

Classic cadherins play critical roles in regulating multiple cellular behaviors in both physiological and pathological conditions, including cell adhesion, cell sorting, cell polarity, invagination, tube formation, cell migration and invasion (Halbleib and Nelson, 2006). Differential expression of cadherins, the so-called “cadherin switch”, is often observed during epithelial mesenchymal transitions (EMT) which involve the morphological conversion of polarized, immotile “epithelial” cell types to more motile/invasive “mesenchymal” cell types.

1.5.2 Cadherins in epithelial-mesenchymal transition and cell invasion

During embryogenesis or carcinoma progression, some cells reduce their adhesive interactions with surrounding cells or ECM, acquire migratory or invasive capabilities, and adapt a different microenvironment. Tissue reorganization and cell movement, accompanied with dynamic changes between arranged epithelial cells and multi-polarized mesenchymal cells, are associated with altered expression of cell adhesion molecules such as cadherins. E-cadherin was first reported to play a critical role in mediating cell-cell adhesion in epithelial cells and is often used as a marker of epithelial cells (van Roy and Berx, 2008). In contrast, N-cadherin and cadherin-11 are highly expressed in motile/invasive cells, and are often detected in cell-cell junctions between mesenchymal
cells, indicating that different cadherin members mediate cell migratory and invasive behaviors via distinct mechanisms.

1.5.2.1 E-cadherin in cell invasion

During early embryo development, E-cadherin is expressed from the first blastocyst stage, where it mediates early cell-cell adhesion during embryo formation. Loss of E-cadherin expression is observed when epiblast cells migrate out during gastrulation. The transcription factor Snail has been implicated as an important mediator of the downregulation of E-cadherin during gastrulation (Carver et al., 2001). Indeed, Drosophila embryos deficient for Snail are unable to form mesenchymal progenitor mesoderm due to their inability to downregulate E-cadherin (Grau et al., 1984).

The expression and function of E-cadherin has been intensively studied in cancer biology since 80-90% of cancers are of epithelial origin. Compromised expression and function of E-cadherin is considered as an important mechanism of certain carcinoma subtype origin. Mutation, epigenetic malfunction, transcriptional silencing are among the various mechanisms involved in E-cadherin dysfunction (Berx and van Roy, 2009). For example, EC domain mutations in E-cadherin which lead to impaired extracellular binding affinity have been detected in gastric cancers (Becker et al., 1993). Moreover, truncated E-cadherin mutations have also been identified in lobular breast cancer cases (Berx et al., 1998). Hypermethylation of a 5’ CpG island in the E-cadherin promoter
region has also been shown to associate with progression of ductal breast carcinoma (Nass et al., 2000).

Aberrant transcriptional regulation induced by EMT-related growth factors has also been shown to correlate with cancer progression. For example, Snail expression is significantly reduced in high grade human ductal breast carcinoma (Blanco et al., 2002). Moreover, another transcription factor ZEB1 promotes tumor cell dedifferentiation by inhibiting epithelial cell polarity and E-cadherin expression (Aigner et al., 2007). Similar pro-invasive effects have also been found for ZEB2 (Comijn et al., 2001; Vandewalle et al., 2005). All the evidences above indicate that different EMT-inducing transcription factors could target the same E-cadherin gene to regulate cell invasive behavior.

1.5.2.2 Mesenchymal cadherins (N-cadherin and Cadherin-11) in cell migration and invasion

The critical roles for mesenchymal cadherins in cell movement were established by studies on gastrulation and neural tube formation. For instance, de novo induction of N-cadherin expression in early gastrulation has been taken as a marker of pro-migratory mesodermal layer formation. Neuronal precursors abundantly express N-cadherin, accompanying with acquired cell migratory capability. N-cadherin expression deficiency in zebrafish leads to impaired migration of neuronal precursors and neural tube formation
(Hong and Brewster, 2006). Moreover, N-cadherin is required for F-actin assembly and cell movement in neural ectoderm (Morita et al., 2010; Nandadasa et al., 2009). Similarly, cadherin-11 is expressed in migrating neural crest cells and is associated with a mesenchymal phenotype during gastrulation (Kimura et al., 1995).

Like its role in embryogenesis, N-cadherin is also associated with cell invasiveness in carcinomas. Elevated expression of N-cadherin is frequently associated with loss of E-cadherin and/or P-cadherin in skin, prostate and breast cancers (Hazan et al., 2004). N-cadherin overexpression provokes cell migration, invasion and metastasis in many cancer models (Hazan et al., 2000; Maret et al., 2010). For example, novel expression of N-cadherin in low invasive bladder cancer cell could provoke its invasiveness in vitro (Rieger-Christ, et al., 2004). Physical interactions with FGF receptor I, leading to activated FGF downstream signaling, have been proposed as a potential mechanism for N-cadherin-induced cell invasion (Kim et al., 2005; Sanchez-Heras et al., 2006).

Aberrant expression of cadherin-11 is associated with increased invasiveness and poor prognosis in a number of tumor types (Bussemakers et al., 2000; Pishvaian et al., 1999; Tomita et al., 2000). For example, overexpression of cadherin-11 promotes cell invasion and metastasis to bone in breast and prostate cancers (Chu et al., 2008; Tamura et al., 2008).
1.5.2.3 Cadherins in angiogenesis

Angiogenesis generally refers to the development of new blood vessels based on an existing vascular network. VE-cadherin plays critical roles in regulating vascular formation and stability (Cavallaro et al., 2006; Dejana, 2004). VE-cadherin in non-endothelial cells not only regulates vascular mimicry, but also affects cell-endothelium interactions. For example, VE-cadherin positive melanoma cells form a vascular-like network, which is disrupted when VE-cadherin is downregulated (Hendrix et al., 2001). N-cadherin is often accompanied by the presence of VE-cadherin in endothelial cells and other cell types. Like VE-cadherin, N-cadherin may also facilitate cell-endothelium interactions. For instance, exogenous expression of N-cadherin in MCF-7 cells enhanced their adhesion to human endothelial cells (Hazan et al., 2000), whereas melanoma cell invasion across an endothelial barrier was inhibited by treatment with N-cadherin function perturbing antibody (Sandig et al., 1997). Moreover, N-cadherin expressing melanoma could penetrate dermal fibroblast and endothelial into circulation by cadherin interaction (Li et al., 2001), and the behavior of melanoma shares large similarity with trophoblast during endovascular invasion. Taken together, there is now clear evidence that cadherins regulate angiogenic phenotypes in addition to their functions in regulating cell-cell adhesion and cell motility.
1.5.3 Expression and function of cadherin in placenta

Various cell types contribute to human placental function: cytotrophoblasts belong to classic epithelia, syncytiotrophoblasts exhibit fused cell morphology, and interstitial EVT s display mesenchymal invasive characteristics and endovascular EVT s exhibit an endothelial-like phenotype. Therefore, the location and expression of different cadherins in the placenta may facilitate their distinct cellular behavior.

E-cadherin has been detected at villous cytotrophoblast and cell column throughout the anchoring villi, and its expression decreases as cells detach from the cell column (Floridon et al., 2000). Isolated cytotrophoblast cells retain E-cadherin expression in vitro (Fisher et al., 1989), where it plays important roles in mediating cytotrophoblast adhesion and invasion (Aplin, 1997).

The cellular localization of N-cadherin in placenta has never been reported before, but is addressed in Chapter IV of my thesis. N-cadherin is produced in primary and immortalized EVT s, where it functions to promote invasion of EVT cells (Ng et al., 2012). Cadherin-11 has been detected at the tips of placental anchoring villi, and is abundantly expressed in EVT s as well as syncytiotrophoblasts (MacCalman et al., 1996; MacCalman et al., 1998). Elevated expression of cadherin-11 in syncytiotrophoblasts is consistent with studies demonstrating its ability to promote primary cytotrophoblast and JEG-3 cell fusion in vitro (Getsios and MacCalman, 2003). In addition, cadherin-11 is upregulated during decidulization suggesting a potential role in mediating trophoblast-decidua
interactions (Chen et al., 1999a).

VE-cadherin does not appear to be expressed in villous cytotrophoblasts, but is expressed in the cell column and by interstitial and endovascular EVTs as well as endometrial endothelial cells (Damsky and Fisher, 1998; Dubernard et al., 2005). VE-cadherin has been reported to promote trophoblastic cell invasion and cell-ECM interaction based on findings that antibodies against VE-cadherin reduce trophoblast adhesion and invasion (Bulla et al., 2005). In addition, VE-cadherin mediates trophoblast-endothelial cell binding in vitro, and reduced VE-cadherin expression in trophoblast has been observed in preeclamptic patients (Damsky and Fisher, 1998; Floridon et al., 2000).

1.5.4 Transcription factor TWIST regulates cadherin expression and cell invasion

TWIST, a member of basic helix-loop-helix (bHLH) transcription factor family, is an evolutionary conserved transcription factor that is found to play important role in both early embryogenesis and pathological diseases.

TWIST is widely expressed in multiple tissues and cell types, especially in mesenchymal cell populations, which processes many migratory characteristics similar with cancer cells during metastasis. Previous studies have revealed that TWIST is over-expressed in metastasis stages in both human and murine cancer (Puisieux et al.,
2006), indicating that TWIST may play crucial role in EMT during cancer progression rather than initiation. For example, depletion of TWIST was reported to prevent lung cancer metastasis, but failed to affect primary tumor formation (Yang et al., 2004). TWIST regulates cancer cell invasion and metastasis through many pathways, and transcriptional suppression of E-cadherin expression may serve as an important mechanism of TWIST induced tumor progression (Vesuna et al., 2008). Twist can form functional homodimers as well as heterodimers with other bHLH transcription factors and inhibit E-cadherin expression through direct binding to E-boxes at the E-cadherin promoter region (Peinado et al., 2007).

Besides E-cadherin suppression, TWIST was also reported to activate the expression of mesenchymal molecules such as vimentin and N-cadherin (Yang et al., 2004). For instance, integrin-induced Twist nucleus translocation could up-regulate N-cadherin gene expression in prostate cancer cells (Alexander et al., 2006). Additionally, TWIST could inhibit other oncogene such as P53- and Rb-induced apoptosis and extending the life-span of tumor cells (Ansieau et al., 2008), which may also lead to an pro-oncogenic effect. TWIST has been detected in invasive EVT cells and it is believed that TWIST could potentiate trophoblast invasion (Ng et al., 2012).
1.6. Proteolysis in trophoblast invasion

1.6.1 Matrix metalloproteinase (MMP)

MMPs, together with ADAMs (a disintegrin and metalloproteinase) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs), comprise the major types of ECM modifying extracellular proteinases. These proteinases are capable of degrading many ECM components such as fibronectin and lamnin. MMPs contain calcium binding sites and are zinc-dependent during enzymatic activation. Twenty three MMP members have been identified in both human and mouse (Jackson, Nebert and Vasilious, 2010).

MMPs are generally subdivided into two groups; membrane-anchored MMPs (MMP14-17 and 23-25) and secreted MMPs (MMP1-3, 7-13, 19-22, 27 and 28). The structures of MMPs can be divided into several different subdomains: a “minimal domain” containing disposable signal sequences and cleavage site, as well as a zinc binding catalytic region; a hinge domain and an Ig-like hemopexin domain. Membrane-anchored MMPs possess either a glycosylphosphatidylinositol anchor or an amino-terminal signal anchor for membrane tethering (Fig. 1.3) (Page-McCaw et al., 2007).

The functions of MMPs have been extensively studied in embryogenesis, cancer
biology, bone remodeling and wound healing. Additional functions include regulating apoptosis, organ morphogenesis, inflammation, immune response, ovulation, endometrial cycling and nerve growth (Verma and Hansch, 2007). Importantly, MMPs are well known regulators of cell invasion and angiogenesis, which are essential for early implantation and placentation.

Figure 1. 4 Structure and subdomains of MMP family members.
1.6.2 MMPs in cell migration and invasion

Due to their ability to degrade ECM proteins, MMPs are well-recognized as essential regulators of cell movement, especially cell invasion. MMPs play important roles in regulating bone remodeling, where MMP9-mediated galectin-3 cleavage is required for osteoprogenitor cells to become migratory and differentiate into osteoblast cells (Vu et al., 1998). In wound healing, MMP7 mediates epithelial cell migration, in part, via cleavage of E-cadherin (McGuire et al., 2003). Moreover, wounds of Mmp-3 mutant mice heal significantly slower compared to wild-type mice, possibly due to impaired cellular motility (Bullard et al., 1999).

Aberrant expression of MMPs is an important mechanism by which tumors acquire metastatic potential. For instance, MMP7 located at the tumor-bone interface induces osteolysis and prostate cancer metastasis in a mouse model (Lynch et al., 2005). Reduced breast cancer bone metastasis has also been observed when MMP1 is knockdown in human breast cancer cell line MDA-MB-231 (Lu et al., 2009). High-resolution microscopy has demonstrated direct pericellular proteolysis of ECM proteins by MMP1, 2, 13 and 14 (Friedl and Wolf, 2008; Wolf et al., 2007). Moreover, elevated MMP2 and MMP9 protein expression have also been detected in patients with high invasive endometrial carcinoma (Weigel et al., 2012). In the cellular level, growth factors such as FGF-2 induced MMP2 and MMP9 directly, contributing to high myoblast invasiveness in vitro (Allen et al., 2003).
1.6.3 MMPs in blood vessel remodeling

Multiple defects in postnatal angiogenesis and vascular development have been observed in a number of MMP mutant mouse models, although primary embryonic vascular development is not affected (Page-McCaw et al., 2007). For example, \textit{Mmp-9} and \textit{Mmp-14} deficient mice present insufficient vascular invasion in cartilage, although VEGF and its receptor level remain unchanged (Vu et al., 1998; Zhou et al., 2000). MMP14 and MMP9 could regulate vascular smooth muscle cell architecture and regulate vascular invasion of tumor cells (Chantrain et al., 2004; Filippov et al., 2005). Moreover, \textit{de novo} vasculogenesis after retina capillary ablation is significantly impaired in \textit{Mmp-2} and \textit{Mmp-9} mutant mice, with more severe defects in \textit{Mmp-2} and \textit{Mmp-9} double mutants (Lambert et al., 2003).

Potential mechanisms involved in MMP-mediated angiogenesis include modulation of platelet-derived growth factor and VEGF signaling, regulation of collagen degradation, and modification of perivascular cells. For example, a physical interaction between MMP14 and platelet-derived growth factor receptor-β leads to activation of pro-angiogenic signaling (Lehti et al., 2005). Moreover, MMP14 and MMP9 could regulate vascular smooth muscle cell architecture and regulate vascular invasion of tumor cells (Chantrain et al., 2004; Filippov et al., 2005). MMPs have also been shown to mobilize or cleave VEGF which could further lead to an increase of vascular protrusion (Bergers et al., 2000).
1.6.4 Expression and function of MMPs in placenta

A number of studies have investigated the roles of MMPs in trophoblast biology, especially in relation to the regulation of trophoblast invasion (Cohen and Bischof, 2007; Cohen et al., 2006). MMPs are secreted by trophoblast cells and regulate ECM dynamics when EVT invades from anchoring villi to endometrium.

Among all the MMPs, MMP2 and MMP9 are the most-studied in first trimester human placenta. MMP2 mRNA and protein has been detected in the cell column region of anchoring villi, with strong immunoreactivity at the distal end where cell invasiveness is highest (Huppertz et al., 1998). MMP2 is abundant in early pregnancy but drops from week 6 to week 11 (Xu et al., 2000). At term, MMP2 levels are detectable but its proteolytic activity appears to be absent (Isaka et al., 2003). MMP9 is detected in both villous cytотrophoblasts and extravillous trophoblasts in the cell column. In early gestation (6-8 weeks) the proteolytic activity of MMP9 equal to that of MMP2, but at weeks 9-12 the activity of MMP9 is greater than that of MMP2 (Staun-Ram et al., 2004).

Membrane-tethered MMPs (MT-MMPs), MMP14 and MMP15, are also expressed in first trimester placenta (Bjorn et al., 2000). Additionally, MMP26 is capable of regulating MMP9 activity in trophoblastic cells (Cohen et al., 2006).
1.6.5 Transcription factor RUNX2 in cell invasion and proteolysis

The mammalian RUNX2, a runt-related transcriptional factor, belongs to a family of transcription factors which are homologues of Drosophila runt. Similar with two other RUNX family members RUNX1 and RUNX3, RUNX2 regulates early developmental related gene expression by forming a heterodimer with co-activator core-binding factor β (Cbfβ) (Kundu et al., 2002; Yoshida et al., 2002). RUNX2 was originally reported as the critical regulator of bone formation and osteoblast differentiation (Komori et al., 1997). Later research further revealed additional roles of RUNX2 in modulating mesenchymal commitment, chondrocyte elongation and vascular permeable into growing skeleton (Otto et al., 1997; Stricker et al., 2002).

Increasing evidence has revealed the roles of RUNX2 in cell migratory and invasiveness, especially in cancer cell metastasis. For example, up-regulated RUNX2 expression has been found in invasive breast cancer and prostate cancer cells compared to low invasiveness carcinomas (Akech et al., 2010; Nagaraja et al., 2006). Moreover, suppression of endogenous RUNX2 expression could reduce prostate cancer aggression \textit{in vitro} (Chua et al., 2009).

RUNX2 has been reported to regulate proteolytic molecules expression in osteoblasts and neoplastic cells, which may contribute to RUNX2-mediated cell invasion. For instance, RUNX2 could upregulate MMP9 and MMP13 expression and promote
osteoblasts migration during bone development (Pratap et al., 2005; Wang et al., 2004). MMP9 is also a major downstream target of RUNX2 during prostate cancer invasion (Akech et al., 2010).

No previous report has reveal the expression and function of RUNX2 in placenta and the regulatory role of RUNX2 in proteolytic proteins expression in placenta is yet to be determined.

1.7 Hypothesis and rationale

The expression of GnRH I, GnRH II, and their mutual receptor (GnRHR) is indicative of potential mechanisms that occur during the dynamic process of human placenta formation and differentiation, particularly during the development of an invasive phenotype of extravillous trophoblasts (EVTs). GnRH has also been reported to promote primary EVT invasion in vitro (Chou et al., 2003). The EMT-related transcription factor TWIST has been shown to up-regulate N-cadherin expression and is associated with pro-invasive phenotypes (Yang et al., 2007). TWIST promotes trophoblast invasion via upregulation of N-cadherin (Ng et al., 2012). Based on previous studies and our preliminary findings, I hypothesize that the regulatory effects of GnRH I and GnRH II are, in part, mediated by their ability to increase N-cadherin expression via TWIST. In this study, I will validate the pro-invasive effect of GnRH in an immortalized extravillous trophoblast cell line. I also will characterize the capability of GnRH in inducing TWIST
as well as N-cadherin expression. Finally, I will examine the involvement of TWIST and N-cadherin in regulating trophoblast invasion with the presence of GnRH.

Cadherin-11 has been reported to positively regulate invasiveness in several cancer cell lines (Huang et al., 2010; Pishvaian et al., 1999). Abundant cadherin-11 signals can be detected at the cell column region of first-trimester placenta villi (MacCalman et al., 1996). Based on our previous observation that GnRH potentiates EVT invasion, here I hypothesize that GnRH is capable to induce cadherin-11 expression in trophoblastic cell. In this study, I will study the capability of GnRH to induce cadherin-11 expression in EVT cell, and will examine the involvement of transcription factors c-FOS and c-JUN in this process.

MMP-2 and MMP-9 are abundantly detected in the first-trimester human placenta and have been documented to play critical roles in regulating cell invasion (Cohen et al., 2006). A RUNT- related transcription factor, RUNX2, was reported to regulate MMP2/9 expression in several other cell types (Akech et al., 2010; Pratap et al., 2005). Moreover, GnRH has been shown to stimulate MMP2 and MMP9 expression in many other cell types, including trophoblast (Poon et al., 2011, Chou et al., 2003). Thus, I hypothesized that GnRH regulates MMP2/9 expression by inducing RUNX2 expression. In this study, I will study the cellular localization of MMP2, MMP9, and RUNX2 in the first-trimester human placenta. I will examine the activation of RUNX2 induced by GnRH in trophoblastic cells. Furthermore, I will investigate the involvement of RUNX2 in GnRH-regulated MMP2/9 expression and trophoblast invasion.
Vascular remodeling by EVT cells is critical to early implantation and placentation. Mesenchymal cadherins and MMPs have been reported to regulate cell angiogenesis and vasculogenesis in endothelial cells (Blaschuk and Rowlands, 2000; Giannopoulos et al., 2008). GnRH has been reported to potentiate angiogenesis in ovary (Parborell et al., 2007), and stimulates pro-angiogenic cytokine secretion in trophoblastic cells (Cavanagh et al., 2009). Here, I hypothesize that GnRH potentiates the trophoblast vascular remodeling process, likely by its regulatory role of modulating cadherin and MMP expression. In this study, I will examine the pro-angiogenic role of GnRH in the immortalized EVT cell line HTR-8/SVneo. I will further investigate the involvement of N-cadherin, cadherin-11, MMP2, and MMP9 in basal and GnRH-regulated tube formation.
CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Tissues

First-trimester human placentas (6-12 weeks) were obtained from women who underwent elective termination of pregnancy under supervision by the Children’s and Women’s Research Ethics Board in the University of British Columbia. All the patients provided their consent and were informed about the purpose of this study.

Human skeletal muscle tissue sections were purchased from Abcam (Cambridge, MA), and mouse muscle tissue was isolated from female wild-type B9 strain mice at age 6-8 weeks after sacrifice.

2.2 Cells

2.2.1 Primary culture of EVT cells

Isolating EVT cells from the first-trimester human placenta has been previously described (Chou et al., 2003c). Briefly, placenta tissues were washed three times in sterile Dulbecco's phosphate-buffered saline (DPBS) (Thermo Scientific, Waltham, MA). Chorionic villi tips were separated from stem villi and minced into fine particles before
being transferred to a Falcon tube. The supernatant containing floating chorionic
membrane debris was removed after incubating the tube for one min. The remaining
placenta villi fragments were plated in 25-cm² tissue culture flasks supplemented with
dulbecco's Minimum Essential Medium (DMEM) (Life Technology, supplemented with
10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) and
were maintained in a 37°C incubator with 5% CO₂. After 1-3 days, nonadherent tissues
were removed, and the villous explants were cultured for another 1-2 weeks before the
outgrowth EVT cells were passaged from chorionic villi by 0.25% trypsin-EDTA (Life
Technology, Carlsbad, CA) digestion. The EVT cells were maintained under the same
culture condition as mentioned above. The purity of the cells was examined by
immunocytochemistry staining of cytokeratin 7. Only cultures that contained more than
99% cytokeratin 7-positive EVTs were used in the following experiments.

2.2.2 Primary culture of human granulosa cells

Primary-cultured human granulosa cells were collected from the IVF Program at the
University of British Columbia. The isolation and primary culture methods used for the
granulosa cells were previously described (Lie et al., 1996). Isolated granulosa cells were
cultured in M-199/MCDB 105 (1:1 mixture) with 10% FBS and maintained at 37°C with
5% CO₂ in air.
2.2.3 Cell lines

The immortalized extravillous trophoblastic HTR-8/SVneo cell line was a kind gift from Dr. P.K. Lala (The University of Western Ontario, London, Ontario). Human chorionic carcinoma BeWo, JAR, JEG-3 cell lines and human liver hepatocellular carcinoma cell line HepG2 were purchased from American Type Culture Collection (Manassas, VA). The HTR-8/SVneo, JAR, JEG-3 and HepG2 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The BeWo cells were maintained in DMEM/F-12K 1:1 with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). All cells were maintained at 37°C in a humidified environment with 5% CO₂ in air.

2.3 Reagents and antibodies

Native human GnRH I, GnRH II, and the GnRH antagonist, Antide, were purchased from Bachem (Belmont, CA). The phosphoinositide3-kinase inhibitor LY294002 and mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 were obtained from Sigma (St. Louis, MO). MMP-2 inhibitor I, MMP-9 inhibitor II and MMP-2/-9 inhibitor I was purchased from Millipore (Billerica, MA). Growth factor reduced Matrigel and
rat-tail collagen I were purchase from BD Biosciences (Franklin lakes, NJ). All the antibodies that were used in this thesis were listed in Table 2.1.

Table 2.1 A list of antibodies used in western blot, immunohistochemistry and immunofluorescence studies in this thesis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody types and species</th>
<th>Supplier/ Clone No./ Catalog No.</th>
<th>Approaches and concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnRHR</td>
<td>Mouse monoclonal purified IgG</td>
<td>Thermo Scientific/Clone F1G4</td>
<td>IHC 10µg/ml Western Blot 0.2µg/ml</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Mouse monoclonal purified IgG</td>
<td>Millipore Upstate/Clone 13A9</td>
<td>IHC 10µg/ml Western Blot 1µg/ml</td>
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2.4 Immunohistochemistry and immunocytochemistry

First-trimester human placentas were fixed in 4% formaldehyde and embedded in paraffin for sectioning. The placenta sections were de-paraffinized three times in 100% xylene for 5 min each. The sections were rehydrated in gradient ethanol solutions (100%, 95%, 80% and 50%) for 3 min each. The sections were incubated in Dako antigen retrieval reagent (pH=6.0) and heated to 100°C for 20 min to expose surface antigen. Primary EVTs, HTR-8/SVneo cells, primary granulosa cells, and HepG2 cells were seeded onto coverglasses and fixed in cold methanol for 20 min before blocking. The sections and coverglasses were incubated for 1 hour in room temperature with serum-free protein block (Dako, Burlington, Ontario, CA) following endogenous peroxidase blocking. The sections were incubated with antibodies against GnRHR, TWIST, N-cadherin, RUNX2, MMP-2 or MMP-9 overnight at 4°C. The concentration of antibodies used in this study is listed in Table 2.1. A universal Dako-labeled streptavidin biotin horseradish peroxidase (HRP) system (Universal LSAB+ Kit/HRP) was used for primary antibody conjugation. The sections were then exposed to a chromagen reagent.
(0.05% diaminobenzidine and 3% H₂O₂) for 5 min for signal detection and counterstained with Harris hematoxylin (Sigma, St. Louis, MO). The sections were observed under a light microscope (Leica, Wetzlar, Germany).

2.5 Immunofluorescence

First-trimester placenta samples were embedded in optimal cutting temperature compound and were rapidly frozen in liquid nitrogen before sectioning. Placenta sections were fixed in cold acetone before immunofluorescence staining. HTR-8/SVneo cells were seeded on coverslips 24h before GnRH treatment. Cells were fixed in cold methanol for 20m before staining. Specific rabbit p-c-FOS, p-c-JUN, c-FOS, c-JUN and mouse HLA-G, α-tubulin antibodies were used for target molecule detection. Cells and placenta sections were incubated with anti-rabbit antibodies conjugated with Alexa Fluo 594 dye and anti-mouse antibodies conjugated with Alexa Fluo 488 dye 1h for signal detection. DAPI was used for nucleus labeling. The sections were observed under a confocal microscope or an upright fluorescence microscope (Leica, Wetzlar, Germany).

2.6 Experimental cell culture

HTR-8/SVneo cells (1×10⁵) were cultured in fixed concentrations of GnRH (100 nM) for different time periods (0, 12, 24, 48, and 72 h) or in increasing concentrations of
GnRH (0, 1, 10, or 100 nM) for 24 h. A shorter series of time points (0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 6 h, and 24 h) was used for cell signaling studies with the presence of GnRH I or GnRH II (100 nM). DNAse and RNAse free water was used as the vehicle control for GnRH. Additionally, different concentrations of Antide (0, 1, 10, or 100 nM) were added to the HTR-8/SVneo cell cultures 1 h prior to GnRH I and II (100 nM) treatment. Cell signaling related kinase inhibitors and MMP inhibitors were added to the cell cultures 30 min before treatment of 100nM GnRH. DMSO were used as the vehicle control for Antide, kinase inhibitors and MMP inhibitors. The primary EVT cells \(2 \times 10^5\) were treated with 100 nM of GnRH I or GnRH II for 24 or 48 h. The culture medium containing GnRH was refreshed every 12 h.

2.7 RNA extraction and first-strand cDNA synthesis

The cells were lysed, and total RNA was extracted using TRIzol reagent (Life Technology, Carlsbad, CA) following the manufacturer’s instructions. RNA concentrations were measured by absorbance at a 260-nm wavelength using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen, Mississauga, ON), and 1 μg of total RNA was used according to the manufacturer's instructions.
2.8 Semi-quantitative and quantitative real-time PCR

Primer sets for RUNX2 (forward: 5’-AGTCCGCAGTCTTTACGAG, reverse: 5’-GCAGAGGTGAGGATGG) and GAPDH (forward: 5’-ATGTTGT-GATGAGTGTGAACCA, reverse: 5’-TGGCAGGTGTTTAGACGGCAG) were used to perform semiquantitative PCR reactions to amplify cDNA from the reverse transcription products. In total, 27-30 amplification cycles were performed to create a detectable yield of amplification product. PCR product aliquots were loaded in a 1% agarose gel to perform electrophoresis. Ethidium bromide was used to stain and visualize the DNA amplification products. The density of staining was quantified using GeneTools software (Syngene, Frederick, MD), and GAPDH was used as a reference.

Real-time qPCR was performed using the ABI Prism 7300 Real-time PCR system (PerkinElmer Applied Biosystems, Foster City, CA) in a 96-well optical microplate. Each 25-μl reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 300 nM of each specific primer, and 25 ng of cDNA. The qPCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 55°C for 1 min. The nucleotide sequences of the resultant PCR products were confirmed by sequencing. The amplification efficiency was determined by plotting log cDNA dilution against ΔCt (ΔCt =Ct.target-Ct.gapdh), the slope of which was close to zero, indicating maximal and similar efficiency of the target and reference genes. Three separate experiments were
performed on different cultures, and each sample was assayed in triplicate. A mean value was used to determine the mRNA levels by the comparative \( C_t (2^{-\Delta\Delta C_t}) \) method with GAPDH as a reference.

2.9 Western blot analysis

The cells were washed twice in cold PBS before cell lysate extraction. The Cell Extraction Buffer (Life Technology, Carlsbad, CA) was used according to the manufacturer’s manual. The supernatant was collected after centrifuging at 15,000g for 15 min. Protein concentrations were quantified using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). An equal amount (30 µg) of protein was electrophoresed on 8% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were immunoblotted with target specific primary antibodies overnight at 4°C. The specific primary antibodies used in western blot are listed in Table 2.1. The signals were detected with the Pierce enhanced chemiluminescence system (ECL, Thermo Scientific, Waltham, MA) after incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To standardize the amounts of protein loaded into each lane, the blots were reprobed with a polyclonal anti-rabbit antibody directed against human β-actin (Sigma Aldrich, St. Louis, MO).
2.10 Small-interfering RNA tranfection

ON-TARGETplus siRNAs (Thermo Scientific, Waltham, MA) targeting human GnRHR, TWIST, N-cadherin, RUNX2, MMP-2, MMP-9, c-FOS, c-JUN and cadherin-11 were transfected into HTR-8/SVneo cells (25 nM siRNA) using Lipofectamine® RNAiMAX Reagent (Life Technology, Carlsbad, CA). The concentration of siRNA used for primary EVT and granulosa cell was optimized to 50nM. In experiments with siRNA transfection in combination of GnRH treatment, GnRH (100 nM) was added to HTR-8/SVneo cell cultures 24h after siRNA transfection. Cells were collected for mRNA and protein at 24h and 48h after GnRH treatment, respectively. Invasion assay and tube formation assay were performed at 24h after GnRH treatment. The cells transfected with ON-TARGETplus control siRNA were used as a negative control in these studies. The culture medium containing GnRH was refreshed every 12 h.

2.11 Matrigel and rat tail collagen I-mediated Transwell invasion assay

The Matrigel and rat tail collagen I-mediated Transwell invasion assay was used to examine cell invasion in vitro. Boyden chambers contain polycarbonate membranes at the bottom with 8µm pore size were used in my study. The HTR-8/SVneo cells were suspended in DMEM containing 0.1% FBS and seeded into growth factor-reduced Matrigel (0.5mg/ml) or acid-extracted rat tail collagen I (0.1mg/ml) (BD Biosciences,
Franklin, NJ) pre-coated Boyden chambers at a density of $1.5 \times 10^4$ per well. The chambers were placed in 12 well plates with 1ml DMEM containing 10% FBS in each well. The plates were incubated in 37°C for 24h to allow the cells to penetrate the Matrigel or collagen barrier. The cells that penetrated the bottom membrane and appeared in the outer surface of Boyden chambers were fixed. The hematoxylin quick stain system (Sigma Aldrich, St. Louis, MO) was used to stain these cells. Non-invaded cells at the inner surface were removed with a cotton swab. The membranes were cut off and mounted on slides, and the cells from five randomly selected areas on each membrane were counted.

2.12 Tube formation assay

The Matrigel-based tube formation assay has been previously described (Arnaoutova et al., 2009; Ponce, 2009), and minor modifications to this protocol were implemented in my experiment. Briefly, growth factor-reduced Matrigel was thawed at 4°C overnight for complete liquidation. Pre-chilled 96-well plates were coated with undiluted Matrigel (10mg/ml, 50µl/well) and were incubated at 37°C for 1 h to enable sufficient gelation. The HTR-8/SVneo cells with or without GnRH treatment were placed on top of the Matrigel at a concentration of 20,000 cells/well. The cells were incubated at 37°C overnight before being plated under an inverted light microscope (Leica, Wetzlar, Germany) for observation. Images covering the entire tube network in each well were
obtained, and the capillary-like tube network was analyzed using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON). The quantifications of the total branch point, total network number, and total tube length were used as indicators of tube formation capability. Three parallel repeats were included in each test, and each experimental condition was repeated on three independent occasions.

2.13 Statistical analysis

At least three independent repeats were performed for each experimental condition. Data on GnRH-induced N-cadherin, TWIST mRNA/protein expressions and HTR-8/SVneo cell invasion (n=10-12) are pooled to be used in normality test. Two different normality tests (D'Agostino's K-squared test and the Shapiro–Wilk test, minimum n=7 required) are used to test how likely are these data set to be normally distributed. All the pooled data passed the normality test and these data are more likely to be normal distributed.

The Student-t test or One-way ANOVA followed by Tukey's test was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA) to analyze the differences between different groups. The differences were considered significant when P < 0.05. The results in the bar graphs are presented as the mean ± S.E.M.
CHAPTER 3: EXPRESSION AND LOCALIZATION OF GnRHR IN FIRST-TRIMESTER HUMAN PLACENTA AND EVT CELLS

3.1 Introduction

As a key regulator of the reproductive system, the primary function of hypothalamic GnRH is to regulate FSH and LH production. GnRH is secreted in a pulsatile manner and binds to its receptor, which is abundantly expressed in pituitary gonadotrophic cells. In addition, growing evidence suggests that GnRH exerts various effects in extra-pituitary tissues (Fraser et al., 1986; Ramakrishnappa et al., 2005; Sharpe, 1980). GnRH has long been detected in ovary (Minaretzis et al., 1995) and it has been shown to suppress steroidogenic enzymes, such as 3-β-hydroxysteroid dehydrogenase and P450 cholesterol side-chain cleavage enzyme activation, leading to a decrease in the progesterone secretion in human granulosa-lutein cells (Sridaran et al., 1999a; Sridaran et al., 1999b). This evidence suggests the existence of GnRH-GnRHR machinery in various peripheral tissues.

The capability of GnRH secretion in peripheral tissues has been previously reported. In addition to the hypothalamus, GnRH I mRNA is also present in several normal human reproductive tissues, such as the ovary, placenta, endometrium, and fallopian tubes, during the luteal phase of the reproductive cycle (Cheng and Leung, 2005). GnRH I
immunoreactivity is also detected in the endometrial stroma, decidua, mononucleated villous cytotrophoblast, and EVTs (Cheng and Leung, 2005; Chou et al., 2004). Moreover, as a second form of GnRH, the conserved GnRH II sequence has been well characterized and is localized in a broad range of non-pituitary tissues and organs. GnRH II mRNA has been detected in the bone marrow, kidney, endometrium, placenta, ovary, mammary gland, and prostate (Millar, 2003, 2005; Sealfon et al., 1997). Approximately 30-fold higher expression of GnRH II has been observed in the kidney, with up to a 4-fold difference in the bone marrow and prostate compared with GnRH I (White et al., 1998). This may indicate that GnRH II exerts predominant effects in peripheral tissue, which are distinct from the well-established neuroendocrine functions of GnRH I. Similar to GnRH I, immunoreactivity for GnRH II is detected in both villous cytotrophoblasts and the EVTs (Chou et al., 2004).

In peripheral organ, the regulatory role of GnRH largely depends on the wide distribution of GnRHR in extra-pituitary tissues. For example, early research using a radioactive-labeled ligand binding assay demonstrated the presence of GnRHR on granulosa cells (Clayton et al., 1979) and Leydig cells (Clayton et al., 1980; Lefebvre et al., 1980). Moreover, the abundant expression of the GnRH receptor in the human placenta and placenta cells has been demonstrated using a variety of approaches (Cheng et al., 2000; Currie et al., 1981; Lin et al., 1995). For example, $^{125}$I-labeled GnRH agonist and native GnRH are capable of binding to placental membrane isolated from term human placenta. Membrane preparations from first trimester hydatidiform mole also
shows a similar binding capacity to radiolabeled GnRH (Currie et al., 1981). An in situ hybridization study revealed that mRNA encoding human GnRHR is localized in both the syncytiotrophoblast and cytotrophoblast layers, and its expression varies from early pregnancy until term, with a peak at 9 weeks (Lin et al., 1995). Moreover, full-length GnRH cDNA has also been isolated from primary cultured first-trimester cytotrophoblasts, immortalized EVTs, and the choriocarcinoma cell line JEG-3 (Cheng et al., 2000). However, the cellular localization of GnRHR protein in the human placenta and trophoblastic cells is still unclear. The purpose of the present study was to examine the expression and localization of GnRHR protein in the first-trimester human placenta and EVT cells using a specific anti-GnRHR antibody.

3.2 Results

3.2.1 GnRHR is expressed in human primary granulosa cells but not in human skeletal muscle

A monoclonal anti-GnRHR antibody (Clone F1G4) was initially used in my immunohistochemistry study to examine the expression and localization of GnRHR in the human placenta. Therefore, the specificity of this antibody needed to be validated. GnRHR mRNA was detected in human granulosa cells aspirated from preovulatory
folicles using RT-PCR; however, the GnRHR-specific primer sets did not amplify the target product from human skeletal muscle (Peng et al., 1994). Moreover, GnRHR mRNA has also been detected in HepG2 human hepatocarcinoma cells (Yin et al., 1998). To validate the specificity of the GnRHR antibody (Clone F1G4), I examined the immunoreactivity for GnRHR in primary human granulosa cells, HepG2 cells, and human skeletal muscle sections. Here, I detected positive immunoreactivity in primary granulosa cells and HepG2 cells but not in skeletal muscle (Fig. 3.1, A, C, and E). No staining was observed in vehicle control sections incubated with mouse IgG instead of GnRHR antibody (Fig. 3.1, B, D, and F).

3.2.2 GnRHR is expressed in mouse pituitary cells but not in mouse skeletal muscle

GnRHR antibody (F1G4) was raised against a synthetic peptide aa1-29 (MANSASPEQNQNHCAINSPLMQGNLPY) from the N-terminal of human GnRH receptor. It shares 90% sequence similarity with mouse GnRHR. To further validate the GnRHR antibody (Clone F1G4) in the detection of mouse GnRHR, I examined the presence of immunoreactivity in the immortalized mouse pituitary cell, LβT2, and mouse skeletal muscle. Intense immunoreactivity was detected in the LβT2 cells but not in mouse skeletal muscle sections (Fig. 3.2, A and C). Negative immunoreactivity was observed in vehicle control sections after substituting the antibody with mouse IgG (Fig. 67
3.2, B and D).

3.2.3 GnRHR is expressed in both primary and immortalized EVT cells

Having validated the immunohistochemistry method to detect human and mouse GnRHR using the GnRHR-specific antibody (Clone F1G4), I then examined the expression levels of GnRHR in the HTR-8/SVneo, primary granulosa, and HepG2 cells by performing Western blot analysis. I detected a single band between 50 and 75 kDa in all 3 cell lines, corresponding to the predicted molecular weight of GnRHR (68 kDa). The HTR-8/SVneo cells presented the lowest expression level among the three cell lines, with four-fold higher expression in the HepG2 cells and ten-fold higher expression in the primary granulosa cells (Fig. 3.3, A). Additionally, the specificity of the antibody was validated by knockdown of GnRHR with specific siRNA in both the primary granulosa cells (Fig. 3.3, B) and HTR-8/SVneo cells (Fig. 3.3, C).

I further investigated the localization of GnRHR in the HTR-8/SVneo cells and primary EVTs using immunohistochemistry. Immunoreactivity was detected in both the HTR-8/SVneo cells (Fig. 3.4, A) and primary EVTs (Fig. 3.4, C). The absence of staining was observed in the vehicle control sections incubated with mouse IgG instead of GnRHR antibody (Fig. 3.4, B and D).
3.2.4 **GnRHR is expressed in the first-trimester human placenta**

The expression of GnRH I, GnRH II and GnRHR in primary and immortalized EVTs strongly suggests that the GnRH-GnRHR system is located at invasion sites in first-trimester placental villi, where EVTs undergo invasive differentiation. Therefore, I examined the localization of GnRHR in first-trimester human placenta using immunohistochemistry. Abundant immunoreactivity was detected in EVT cells located in the cell column region, especially in the distal zone of the cell column (Fig. 3.5, A). Moreover, GnRHR staining was also observed in the cytotrophoblast layer of the first-trimester human placenta (Fig. 3.5, A). Negative staining was observed in vehicle control sections after substituting anti-GnRHR antibody with mouse IgG during incubation (Fig. 3.5, B).

3.3 **Discussion**

Peripheral distribution of GnRHR mRNA has been investigated in many non-pituitary tissues using RT-PCR and *in situ* hybridization methods (Chen et al., 1999b; Peng et al., 1994; Yin et al., 1998). Early evidence also revealed the presence of GnRHR mRNA in the first-trimester human placenta and placental cells (Cheng et al., 2000). Early studies indirectly established the presence of GnRHR protein in the placenta through the use of ligand-binding assays with placenta- and trophoblast-derived
membrane preparations (Currie et al., 1981; Iwashita et al., 1986). A validated antibody against GnRHR allows for the detection GnRHR protein by immunohistochemistry or Western blot analysis. A GnRHR antibody (Clone F1G4) was previously used to detect the presence of GnRHR in human granulosa cells (Cheng et al., 2002). This antibody was later used to perform immunohistochemical analyses for GnRHR in C. elegans (Vadakkadath Meethal et al., 2006). Here, in my study, I used GnRHR-expressing human primary granulosa cells as a positive control and GnRHR-absent human skeletal muscle as a negative control to validate the sensitivity of Clone F1G4 in the immunohistochemistry experiments. The specific GnRH siRNA strategy was also performed to validate the specificity of this antibody in Western blot analysis. Based on my data, the GnRHR antibody (Clone F1G4) exhibited sensitivity and specificity in recognizing human GnRHR protein.

Serum levels of GnRH and placental GnRH mRNA expression vary little throughout pregnancy, however dynamic changes in GnRHR mRNA levels are observed at different periods of gestation (Lin et al., 1995). GnRHR mRNA is abundant at 6 weeks, reaches its peak at 9 weeks, and declines from 12-20 weeks to complete absence at term (Lin et al., 1995). High GnRHR expression during the first trimester is temporally correlated with robust EVT proliferation and differentiation, suggesting that the EVT cell population may express abundant levels of GnRHR. In support of this, I detected clear GnRHR immunoreactivity in both primary and immortalized EVTs using immunocytochemistry and Western blot analysis. Moreover, immunohistochemistry demonstrated GnRHR in the
cell column region of first-trimester placenta. That GnRHR protein was detected in villous cytotrophoblasts, but not syncytiotrophoblasts, is not consistent with the findings of Lin et al. regarding GnRHR (Lin et al., 1995). Indeed, numerous studies suggest that GnRH stimulates hCG production in trophoblastic cells via GnRHR (Belisle et al., 1984; Butzow, 1982; Khodr and Siler-Khodr, 1978a). The syncytiotrophoblast secretes hyperglycosylated hCG, which is different from choriocarcinoma-originated hCG (Kovalevskaya et al., 2002). The evidence above suggests that distinct mechanisms may be involved in hCG secretion by these two cell types, which may be related to different GnRHR expression levels.

The temporospatial expression of GnRHR in the placenta suggests that local GnRH may regulate early placenta development and function. The abundant GnRHR signaling in the EVT cell population provides evidence that GnRH may regulate trophoblast function via its receptor. In the following chapters, I will discuss the regulatory effect of GnRH in modulating EVT invasion and vascular remodeling, likely through modification of cell adhesion molecules and proteolysis.
Figure 3.1 GnRHR is immunolocalized in primary granulosa cells and HepG2 cells but not in human skeletal muscle tissue.

Primary granulosa cells and HepG2 cells were cultured on top of coverslips and culture for 24h until reaching 50% confluence prior to fixation in cold methanol. Paraformaldehyde fixed and paraffin-embedded human skeleton muscle sections were deparaffinized and rehydrated by serial incubations in xylene and ethanol solution (see Chapter 2 section 2.4). Cells and tissue samples were incubated overnight with primary antibody against GnRHR (A, C and E) or mouse Ctrl IgG (B, D and F) before anti-mouse secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hemotoxylene. The specimens were observed under a light microscope and all experiments were repeated three times. The representative pictures in (A-D) are shown in 400× magnification with scale bar size at 25µM and the pictures in (E-F) are shown in 100× magnification with scale bar size at 100µM. GnRHR immunoreactivity was positively detected in primary granulosa cells (A) and HepG2 cells (C) and no immunoreactivity was observed in human skeletal muscle tissues (E). Primary granulosa cells (B), HepG2 cells (D) and human skeletal muscle tissues (F) incubated with mouse Ctrl IgG were used as blank controls.
Primary granulosa cells

HepG2 cells

Human skeletal muscle
Figure 3. 2 Mouse pituitary LβT2 cells but not mouse skeletal muscle tissue express GnRHR.

LβT2 cells were plated on coverslips and cultured for 24h before prior to fixation in cold methanol. Paraformaldehyde fixed and paraffin-embedded human skeleton muscle sections were deparaffinized and rehydrated by serial incubations in xylene and ethanol solution (see Chapter 2 section 2.4). Cells and tissue samples were incubated overnight with primary antibody against GnRHR (A and C) or mouse Ctrl IgG (B and D) before anti-mouse secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hemotoxylene. The specimens were observed under a light microscope and all experiments were repeated three times. The representative pictures in (A-B) are shown in 400× magnification with scale bar size at 25μM and the pictures in (C-D) are presented in 100× magnification with scale bar size at 100μM. GnRHR immunoreactivity was positively detected in mouse pituitary LβT2 cells (A) and no immunoreactivity was observed in mouse skeletal muscle tissues (C). Mouse pituitary LβT2 cells (B) and mouse skeletal muscle tissues (D) incubated with mouse Ctrl IgG were used as blank controls.
LβT2 cells

Mouse skeletal muscle
Figure 3. Expression of GnRHR in immortalized human EVT cells and the HTR-8/SVneo cell line.

(A) Primary granulosa cells, HTR-8/SVneo cells and HepG2 cells were cultured and whole cell lysates were collected for SDS-PAGE and western blot. Representative chemiluminogram of western blot shows the expression of GnRHR protein in these cells. Primary granulosa cells and HepG2 cells were used as positive controls.

Primary granulose cells and HepG2 cells were transfected with GnRHR specific siRNA or Ctrl siRNA for 48h. Whole cell lysates were collected for SDS-PAGE and western blot. Representative cheniluminogram of western blot shows the specificity of GnRHR antibody in recognizing GnRHR protein in primary granulosa cells (B) or HTR-8/SVneo cells (C) using siRNA strategy. All immunoblots were probed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. In all experiments, three immunolots from independent experiment were scanned and signals were quantified by Genetools software. The quantified values of HTR-8/SVneo in bargraph (A) and Ctrl siRNA in bargraphs (B and C) were normalized to 1. The graphs in the right panel show the mean ± S. E. M. Significant differences between groups are indicated with different letters above each bar (n=3, P<0.05, Tukey test followed by one-way ANOVA (A) or Student-t test (B and C)).
Figure 3. 4 Immunohistochemistry staining demonstrates the localization of GnRHR in primary EVT cells and immortalized HTR-8/SVneo cells.

Primary EVT cells and HTR-8/SVneo cells were plated on coverslips and cultured for 24h until reaching 50% confluence prior to fixation in cold methanol. Cells were incubated overnight with primary antibody against GnRHR (A and C) or mouse Ctrl IgG (B and D) before anti-mouse secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hemotoxylene. The slides were observed under a light microscope and all experiments were repeated three times. The representative pictures are shown in 400× magnification with scale bar size at 25µM. GnRHR immunoreactivity was positively detected in both primary EVT cells (A) and HTR-8/SVneo cells (C). Primary EVT cells (B) and HTR-8/SVneo cells (D) incubated with mouse Ctrl IgG were used as blank controls.
Figure 3. 5 GnRHR is immunolocalized in the EVT cell column region and the mononucleate cytotrophoblast layer in the first-trimester human placenta.

Paraformaldehyde fixed and paraffin-embedded human first trimester placenta sections (week 7-12) were deparaffinized and rehydrated by serial incubations in xylene and ethanol solution (see Chapter 2 section 2.4). Placenta samples were incubated overnight with primary antibody against GnRHR (A) or mouse Ctrl IgG (B) before anti-mouse secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hematoxylene. The specimens were observed under a light microscope and all experiments were repeated three times. The representative pictures are shown in 100× magnification with scale bar size at 100µM.
First trimester human placenta

A
- GnRHR

B
- Ctrl IgG
CHAPTER 4: GnRH REGULATES HUMAN TROPHOBLAST CELL INVASION VIA TWIST-INDUCED N-CADHERIN EXPRESSION

4.1 Introduction

Embryo implantation is a critical step during early pregnancy (Paria et al., 2002), and abnormal implantation directly influences pregnancy outcomes (Norwitz et al., 2001). During early implantation and placentation, a population of cytotrophoblasts enters two diverse pathways to differentiate into two functionally distinct cell types (Red-Horse et al., 2004). Cytotrophoblasts located in the placenta villi fuse into a multinucleate monolayer of syncytiotrophoblasts (Potgens et al., 2002). In contrast, cells derived from columned cytotrophoblasts acquire a highly invasive phenotype and become EVTs (Knofler et al., 2008). The EVT cells that enter the opening of maternal endometrial spiral arteries become endovascular EVTs, and the cells that invade the endometrial stroma layer comprise the interstitial EVT population (Gude et al., 2004). The acquisition of cell invasion is accompanied by dynamic changes in cell adhesion behavior and cell morphology. The cell adhesive and morphological alterations allow cells to detach from the placenta cell column and establish de novo interactions with the endometrial compartment. A series of research studies revealed that adhesion molecules, such as integrins and cadherins, undergo programmed switching during extravillous trophoblast differentiation (Coutifaris et al., 1991; Damsky et al., 1994), demonstrating that this
cellular behavior is tightly regulated, unlike the behavior of cancer cells. Disruption of trophoblast invasion may result in inadequate implantation or failure, which may lead to severe outcomes such as spontaneous miscarriage, intrauterine growth restriction, and preeclampsia (Norwitz et al., 2001). Increasing evidence shows that a variety of growth factors, hormones, and their receptors are detected in extravillous trophoblast cell populations, suggesting that trophoblast invasion is a tightly controlled and multi-factor-regulated process (Anteby et al., 2005; Chou et al., 2004).

The decapeptide GnRH is well known as a central regulatory of the reproductive system by virtue of its key role in regulating the hypothalamus-pituitary-gonad (HPG) axis. However, GnRH and its receptor have also been detected in peripheral organs and many types of cancer (Harrison et al., 2004). These findings suggest that GnRH may directly regulate the physiological and pathological functions of these target tissues. GnRH II has been shown to stimulate cell invasiveness in ovarian cancer cells (Poon et al., 2011); however, the opposite has been reported in breast cancer cells (Aguilar-Rojas et al., 2012) and melanomas (Moretti et al., 2008). Our group has previously demonstrated that GnRH is abundantly expressed in the placenta (Chou et al., 2004), suggesting a role for GnRH as a local regulator of cell behavior during pregnancy. Indeed, we demonstrated that GnRH induces MMP2 and MMP9 expression in EVT cells (Chou et al., 2003c). GnRH has been reported to activate JNK signalling and increase MMP26 expression in EVT cells (Liu et al., 2010), however additional mechanisms mediating the pro-invasive effects of GnRH on trophoblasts have yet to be elucidated.
N-cadherin, a calcium-dependent adhesion molecule, is one of the type I classical cadherins in the cadherin superfamily (Yagi and Takeichi, 2000). This protein was first identified in neuronal synapses and was demonstrated to play an extremely important role in neuronal growth and development (Takeichi, 2007). N-cadherin knockout mice die at an early gestational age due to neural tube and somite developmental failure (Radice et al., 1997). Furthermore, increasing evidence suggests that N-cadherin, a mesenchymal cadherin, has elevated expression and is associated with the cell-invasive phenotype in metastatic cancer. Overexpression of N-cadherin in breast cancer cells was shown to promote cell migration, invasion, and metastasis both in vivo and in vitro (Hulit et al., 2007). The basic helix-loop-helix transcription factor TWIST (also known as TWIST1) is a master regulator of epithelia mesenchymal transition. It controls the down-regulation of epithelial markers such as E-cadherin and β-catenin, and is involved in the up-regulation of mesenchymal markers such as N-cadherin, vimentin, and fibronectin (Yang et al., 2004). In prostate cancer cells, N-cadherin gene expression is modulated by TWIST nuclear translocation (Alexander et al., 2006). Our previous finding also suggests that alternative TWIST expression patterns are relevant to the trophoblast-invasive capacity (Ng et al., 2012).

The purpose of this study was to investigate the involvement of TWIST-mediated N-cadherin expression in GnRH-induced trophoblast invasion. We demonstrate that treatment with GnRH upregulates N-cadherin expression via TWIST, and that both contribute to GnRH-induced cell invasion.
4.2 Results

4.2.1 Co-expression of TWIST, N-cadherin, and GnRHR in first-trimester human placenta

Abundant immunoreactivity for TWIST, N-cadherin, and GnRHR was detected in the cell column of first-trimester placental villi, especially in the distal end of the villi tips. Moderate staining of TWIST, N-cadherin, and GnRHR was observed in the cytotrophoblast layer (Fig. 4.1.A).

4.2.2 Differential expression of TWIST and N-cadherin correlates with cell invasiveness in trophoblastic cells

Highly invasive primary EVTs and HTR-8/SVneo cells displayed much higher levels of TWIST and N-cadherin protein than choriocarcinoma cell lines with low invasive capacity (JAR, JEG-3, and BeWo cells; Fig. 4.1.B and C).
4.2.3 GnRH induces TWIST and N-cadherin expression in both HTR-8/SVneo cells and primary EVTs

Significant increases in TWIST and N-cadherin mRNA and protein levels were observed following treatment of HTR-8/SVneo cells with 100 nM GnRH I or GnRH II for 24, 48 or 72 h (Fig. 4.2.A and B). In concentration-dependent studies (1-100 nM), only 100 nM GnRH I or GnRH II was effective in stimulating TWIST and N-cadherin mRNA and protein production in HTR-8/SVneo cells (Fig. 4.2.C and D).

The regulatory effects of GnRH on TWIST and N-cadherin were also examined in primary EVTs. Significant elevations of TWIST and N-cadherin mRNA levels were observed following treatment for 24 h with 100 nM GnRH I or GnRH II (Fig. 4.2.E and F). Taken together, these results suggest that GnRH stimulates TWIST and N-cadherin mRNA expression in human primary EVTs.

4.2.4 Antide attenuates GnRH-induced TWIST and N-cadherin expression and HTR-8/SVneo cell invasion

To confirm the specificity of GnRH-induced TWIST and N-cadherin expression, HTR-8/SVneo cells were pre-incubated with different concentrations of the GnRHR antagonist Antide (1, 10, and 100 nM) prior to treatment with 100 nM GnRH.
GnRH-induced TWIST (Fig. 4.3 A and B) and N-cadherin expression (Fig. 4.3 C and D), as well as HTR-8/SVneo cell invasion (Fig. 4.3 E), were significantly attenuated by 100 nM Antide.

4.2.5 Loss of TWIST expression reduces basal as well as GnRH-induced cell invasion via the down-regulation of N-cadherin in HTR-8/SVneo cells and primary EVT

Transient siRNA transfection was used to investigate the effects of endogenous TWIST and N-cadherin on trophoblast invasion. Nonsilencing siRNA-transfected HTR-8/SVneo cells served as the control. The invasiveness of the HTR-8/SVneo cells was significantly reduced by knockdown of endogenous TWIST and N-cadherin (Fig. 4.4 A and C, respectively). In addition, significant decreases in N-cadherin protein levels were observed following treatment of HTR-8/SVneo cells with TWIST siRNA (Fig. 4.4.B). Similarly, down-regulation of TWIST reduced N-cadherin expression, and knockdown of either TWIST or N-cadherin significantly reduced cell invasion in primary EVT (Fig. 4.4. D and E).

Importantly, depletion of TWIST attenuated GnRH-stimulated N-cadherin mRNA and protein expression (Fig. 4.5.A and B) as well HTR-8/SVneo cell invasiveness (Fig. 4.5 C).
4.2.6 Loss of N-cadherin expression and function attenuates GnRH-induced HTR-8/SVneo cell invasion

A loss-of-function approach was also used to study the involvement of N-cadherin in GnRH-mediated trophoblast invasion. GnRH-induced N-cadherin expression was completely abolished after transfection of HTR-8/SVneo cells with N-cadherin siRNA (Fig. 4.5.D). Importantly, treatment with N-cadherin siRNA reduced the pro-invasive effects of GnRH on HTR-8/SVneo cells, however this effect was only partial (Fig. 4.5.E).

To further confirm the role of N-cadherin in GnRH-induced trophoblast invasion, we employed an alternative approach using an antibody to block the activity of N-cadherin. Incubation with function perturbing antibody against N-cadherin partially inhibited GnRH-induced HTR-8/SVneo cell invasion was significantly but not completely abolished (Fig. 4.5.F).

4.2.7 Role of PI3K/AKT signaling in GnRH I- and GnRH II-induced TWIST and N-cadherin expression

To examine the involvement of AKT signaling in GnRH-induced TWIST and N-cadherin expression, the HTR-8/SVneo cells were incubated with 100 nM of GnRH for different time periods (5 min, 15 min, 30 min, 1 h, 2 h, and 6 h). Increased levels of
phospho-AKT protein were detected as early as 5 min and lasted up to 2 h after treatment with GnRH I or GnRH II (Fig. 4.6.A).

To determine whether the PI3K/AKT pathway is involved in mediating the effects of GnRH on TWIST and N-cadherin expression, HTR-8/SVneo cells were pre-incubated for 1 h with the PI3K inhibitor LY294002 prior to GnRH treatment. Basal and GnRH-induced TWIST and N-cadherin protein production were reduced by co-treatment with LY294002 (Fig. 4.6.B). These results suggest that the PI3K/AKT pathway is involved in GnRH-mediated TWIST and N-cadherin expression in trophoblastic cells.

4.3 Discussion

The pro-invasive function of GnRH has been extensively studied in many cancer, including ovarian carcinomas (Dabizzi et al., 2003; Ling Poon et al., 2011; Poon et al., 2011). The associated mechanism primarily involves GnRH-regulated matrix metalloprotease (MMP) functions. For example, GnRH II was reported to up-regulate MMP-2 and membrane type I MMP expression as well as promote cell invasion in ovarian carcinomas (Ling Poon et al., 2011; Poon et al., 2011). GnRH I and GnRH II were also reported to promote first-trimester trophoblast invasion by modulating the expression of MMP-2, MMP-9, urokinase-type plasminogen activator (uPA), and its inhibitor PAI (Chou et al., 2003c; Chou et al., 2002).

We now demonstrate that both GnRH I and GnRH II promote cell invasion by
up-regulating N-cadherin in both immortalized and primary cultured EVTs. N-cadherin is a member of the cadherin superfamily and is believed to mediate the cell epithelial-mesenchymal transition. These findings are in agreement with our previous studies on endogenous N-cadherin (Ng et al., 2012), and suggest an important functional role for N-cadherin in basal and growth factor-induced trophoblast cell invasiveness. N-cadherin has been widely reported to mediate cell aggregation and promote invasion and metastasis in various cell types (Hulit et al., 2007; Ng et al., 2012). Pathologically, higher N-cadherin expression was reported to be associated with a poor prognosis; therefore, targeting N-cadherin has been used as a therapeutic approach in metastatic cancer treatment (Mariotti et al., 2007). Physiologically, embryonic N-cadherin controls granule neuron migration and plays critical roles in early nervous system development (Rieger et al., 2009). N-cadherin regulates cell motility primarily by mediating cell contact, modulating Rho GTPase activities, or activating receptor tyrosine kinases (Derycke and Bracke, 2004). For example, N-cadherin was reported to induce cell invasiveness by binding to P120-catenin and triggering RhoA/Rac1 activation in breast and renal cancer cells (Yanagisawa and Anastasiadis, 2006). Interestingly, GnRH was reported to regulate ovarian cancer cell invasion by activating tyrosine phosphorylation of P120-catenin via the mesenchymal cadherin P-cadherin (Cheung et al., 2011). Based on these observations, we hypothesize that the pro-invasive effects of mesenchymal N-cadherin in EVTs, either basal or GnRH-induced, are mediated by its interaction with P120-catenin and related downstream pathways. Further experiments exploring this
hypothesis are still underway.

We also demonstrated the involvement of Twist in GnRH-induced N-cadherin expression. Known primarily as a suppressor of E-cadherin, the EMT-related transcription factor TWIST is also associated with the expression of N-cadherin in embryonic cells and carcinomas (Kang and Massague, 2004). In lung epithelial cells from lung fibrosis patient, increased N-cadherin expression was accompanied by TWIST up-regulation (Pozharskaya et al., 2009). Yang and colleagues observed elevated N-cadherin expression after TWIST over-expression in both MDCK and HMEC cells (Yang et al., 2004). Our IHC results demonstrate a similar localization of these two proteins, particularly at the highly invasive placental villous tip. In addition, N-cadherin levels were tightly correlated with those of TWIST, and both were correlated with the overall invasiveness of primary EVT and four different trophoblastic cell lines. Exactly how TWIST regulates N-cadherin transcription remains unclear, however a putative TWIST binding site has been identified in the first exon of the N-cadherin gene by electrophoretic mobility gel shift assay (Alexander et al., 2006). In addition, a transcriptional coactivator of TWIST, SET8, was shown to be recruited to the N-cadherin promoter and to mediate N-cadherin expression as well as metastasis (Yang et al., 2012).

Many studies have revealed the potential signaling cascades that are directly and indirectly triggered by GnRH in different cell types. By selectively binding to different G proteins, GnRHR activation triggers different canonical downstream cytoplasmic signaling cascades such as the PKC and cAMP pathways (Neves et al., 2002). In addition
to these canonical pathways, GnRHR has also been shown to activate the MAPK, PI3K/AKT, and/or NF-κB pathways, either in pituitary gonadotrophs or extrapituitary tissues (Naor, 2009). Indeed, our group has established that GnRH induces the rapid activation of PI3K/AKT signaling in ovarian cancer cells (Ling Poon et al., 2011). The results presented here are the first to demonstrate the induction of AKT phosphorylation by GnRH in HTR-8/SVneo cells. In addition, we used a PI3K inhibitor to confirm the involvement of PI3K/AKT signaling in GnRH-induced TWIST and N-cadherin expression. Recently, in support of our observations, Pai and colleagues reported that inhibition of AKT phosphorylation, could further reduce TWIST and N-cadherin expression, as well as cell invasion and metastasis, in breast cancer cells (Pai et al., 2013).

Nevertheless, controversy still remains regarding the involvement of the PI3K/AKT pathway in the GnRH and GnRH receptor axis. It has been reported that down-regulation of the phospho-AKT level in αT3-1 cells with the treatment of GnRH (Rose et al., 2004). The different observations of PI3K/AKT activation may be related to the functional variations of GnRH itself in specific cell types. In addition to its pro-invasive roles in EVT and several ovarian cancer cell lines, GnRH was reported to exert an anti-invasive effect on breast cancer cells (Aguilar-Rojas et al., 2012).

In summary, the results presented in this chapter reveal important roles for GnRH I and GnRH II in human trophoblast cell invasion. Both GnRH I and GnRH II up-regulate the expression of Twist, and subsequently N-cadherin, via GnRH receptor-activated PI3K/AKT signaling. These findings have demonstrated the involvement of GnRH and
its receptor in human first-trimester placenta implantation and the possible molecular changes during this critical period. In addition, our data provide strong evidence of the potential neuropeptides involved in molecular mechanisms in human placenta development, which furthers our understanding of the human placentation process as well as maternal-fetal interactions.
Figure 4. 1 GnRHR, TWIST, and N-cadherin are expressed in the first-trimester human placenta and are correlated with trophoblastic cell invasion.

Paraformaldehyde fixed and paraffin-embedded first trimester human placenta sections were deparaffinized and rehydrated by serial incubations in xylene and ethanol solution (see Chapter 2 section 2.4). Tissue samples were incubated overnight with primary antibody against GnRHR, TWIST, N-cadherin or Ctrl IgG before secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hemotoxylene. The specimens were observed under a light microscope and all experiments were repeated three times. Immunohistochemistry staining of TWIST, N-cadherin and GnRHR in first trimester human placenta is shown in the micrographs (A). The micrographs are presented in 100× magnification with scale bar size at 100µM. Primary EVT cells, HTR-8/SVneo cells, choriocarcinoma JEG-3, JAR and BeWo cells were cultured for Matrigel-mediated transwell invasion assay (see Chapter 2 section 2.10) or collected for western blot analysis. TWIST and N-cadherin protein levels in trophoblastic cell lines and primary EVT cells are presented in micrograph (B). The overall cell invasiveness of primary EVT cells and trophoblastic cell lines are presented in bargraph (C). Each experiment was repeated three times with the same condition and all the data are presented as mean ± S.E.M.. Significant different values are indicated with different letters (n=3, P<0.05, Tukey test after one-way ANOVA).
A

Twist

N-cad

GnRHR

Ctrl IgG

B

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<tr>
<th>EVT</th>
<th>HTR-8/SVneo</th>
<th>JAR</th>
<th>JEG-3</th>
<th>BeWo</th>
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</table>

Twist

N-cadherin

β-actin (n=3, P<0.05)

C

Invaded cell number (fold change)

| EVT | HTR-8/SVneo | JAR | JEG-3 | BeWo |

a

b

b

c
Figure 4. 2 GnRH stimulates TWIST and N-cadherin expression in HTR-8/SVneo cells and primary EVTs.

HTR-8/SVneo cells were incubated either with 100nM of GnRH for different time (0h, 12h, 24h, 48h or 72h), or with different concentrations (0nM, 1nM, 10nM, or 100nM) of GnRH for 48h. DNAse and RNAse water was used as the vehicle ctrl for GnRH. The cells are collected for mRNA extraction or SDS-PAGE. The mRNA and protein expression of target genes in these cells were analyzed by qRT-PCR and western blot, respectively. The immunolots were scanned and signals were quantified by Genetools software. The mRNA and protein levels of TWIST after GnRH incubation are presented in bargraphs (A) and (C), respectively. The mRNA and protein levels of N-cadherin in these cells after GnRH treatment are presented in bargraphs (B) and (D), respectively. Primary EVTs were treated with 100nM GnRH and cells are collected at 24h and 48h after treatment. Relative mRNA levels of TWIST and N-cadherin were examined by qRT-PCR and are shown in the bargraphs (E) and (F), respectively. The values of each time point controls in bargraphs (A), (B), (D), (E) and (F) are normalized to 1 and presented as Ctrl. The TWIST and N-cadherin protein levels of HTR-8/SVneo cell in 0h are normalized to 1 and presented as 0h in bargraphs (B). Each experiment was repeated at least three times with the same condition and all data are presented as mean ± S.E.M.. Significant different values are indicated with different letters (n=3, n=5 in primary EVTs, P<0.05, Tukey test after one-way ANOVA).
Figure 4. 3 The GnRH antagonist, Antide, attenuates GnRH-induced TWIST and N-cadherin expression and HTR-8/SVneo cell invasion.

HTR-8/SVneo cells were pre-incubated with different concentrations (0, 1nM, 10nM and 100nM) of Antide 1h before treatment with 100nM of GnRH. DNAse and RNAse free water is used as the vehicle control of GnRH and Antide. The cells were collected at 48h after GnRH treatment for mRNA extraction or SDS-PAGE. The mRNA and protein expression of target genes in these cells were analyzed by qRT-PCR and western blot, respectively. All immunoblots were probed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. The immunolots were scanned and signals were quantified by Genetools software. The expression of TWIST mRNA, TWIST protein, N-cadherin mRNA and N-cadherin protein is presented in the bargraphs (A), (B), (C), and (D), respectively. A single concentration of GnRH (100nM) was used with or without the presence of Antide in transwell invasion assay. The invasiveness of HTR-8/SVneo cells in these cells is presented in the bargraph (E). The mRNA or protein levels of TWIST and N-cadherin with 100nM Antide alone are normalized to 1 in bargraphs (A-D). The invasiveness of HTR-8/SVneo cells without GnRH and Antide is normalized to 1 and presented as Ctrl in bargraph (E). Each experiment was repeated three times and all data are presented as mean ± S.E.M.. Different letters indicate significant differences (n=3, P<0.05, Tukey test after one-way ANOVA).
Figure 4. The loss-of-function of TWIST or N-cadherin attenuates N-cadherin expression as well as trophoblastic cell invasion.

HTR-8/SVneo cells and primary cultured EVT cells were transfected with control siRNA, siRNA against Twist or siRNA against N-cadherin (see Chapter 2 section 2.9). The cells were collected for western blot analysis or trypsinized for Matrigel-mediated transwell invasion assay. The protein level of TWIST and the cell invasiveness of HTR-8/SVneo cells after TWIST siRNA transfection are presented in (A). The relative protein level of N-cadherin in HTR-8/SVneo cells after TWIST siRNA transfection is presented in bargraph (B). The expression of N-cadherin protein and cell invasiveness after loss-of-function of N-cadherin in HTR-8/SVneo cells are shown in (C). The protein levels of TWIST and N-cadherin in primary EVTs after siRNA trasfection are presented in micrograph (D). The cell invasiveness of primary EVTs after TWIST or N-cadherin siRNA transfection is presented in bargraph (E). The invaded cell numbers of HTR-8/SVneo cells after Ctrl siRNA tranfection in bargraphs (A), (C), and (E) are normalized to 1. The relative protein level of N-cadherin after Ctrl siRNA transfection in bargraph (E) is normalized to 1. Each experiment was repeated at least three times (n=3, n=4 in (D) and (E)) and all data are presented as mean ± S.E.M.. Significant different values are indicated with different letters (P<0.05, student t-test in (A), (B) and (C), Tukey test after one-way ANOVA in (D) and (E)).
D

<table>
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<tr>
<th>Ctrl siRNA</th>
<th>siN-cad</th>
<th>siTWIST</th>
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<tbody>
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<td></td>
<td></td>
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<tr>
<td>72h</td>
<td>N-CADHERIN</td>
<td></td>
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<tr>
<td>β-actin</td>
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(n=4, P<0.05)

E

- Invaded cell number (fold change)

- Ctrl siRNA
- siN-cad
- siTwist

Legend:
- a
- b
Figure 4. 5 Knockdown of TWIST or N-cadherin attenuates GnRH-induced N-cadherin expression and cell invasion in HTR-8/SVneo cells.

HTR-8/SVneo cells were incubated with 100nM GnRH from 24h after TWIST or N-cadherin siRNA transfection. DNAse and RNAse free water was used as the vehicle control for GnRH. Cells were either collected after 48h after GnRH treatment or seeded for Matrigel-mediated invasion assay 24h after GnRH treatment. All immunoblots were probed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. The immunoblots were scanned and signals were quantified by Genetools software. In invasion assay, the cells were manually counted under a light microscope. Representative N-cadherin mRNA (A), N-cadherin protein (B) and cell invasiveness (C) after TWIST siRNA transfection are shown in the bargraphs. Representative N-cadherin protein and cell invasion after N-cadherin siRNA transfection are shown in bargraphs (D) and (E), respectively. N-cadherin perturbing antibody was added in to HTR-8/SVneo 24h after GnRH treatment. The cells were placed into boyden chamber for invasion assay 1h after functional perturbing antibody treatment. Mouse IgG was used as control for N-cadherin perturbing antibody. The cell invasiveness of HTR-8/SVneo cells was presented in (F). The values of Ctrl siRNA groups in bargraphs (A), (B), (C) and (E) are normalized to 1. Each experiment was repeated three times (n=3) and all data are presented as mean ± S.E.M.. Significant different values are indicated with different letters (P<0.05, Tukey test after ANOVA).
Figure 4. GnRH stimulates TWIST and N-cadherin expression via the PI3K/AKT signaling pathway.

HTR-8/SVneo cells are incubated with GnRH I and GnRH II and the cells are collected in different time points (5m, 15m, 30m, 1h, 2h and 6h) for SDS-PAGE. AKT and phosphor-AKT protein levels in different time points after GnRH treatment are detected using western blot and are presented in bar graph (A). HTR-8/SVneo cells are incubated with GnRH in the combination of LY294002. DMSO was used as the vehicle control of LY294002. The cells are collected at 48h after GnRH treatment. The protein levels TWIST and N-cadherin are examined using western blot and are presented in micrograph (B). All the immunoblots were reprobed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. Each experiment was repeated three times (n=3, P<0.05, Tukey test after one-way ANOVA).
A

\[
\begin{array}{c|ccccccc}
& \text{GnRH I 100nM} & & & & & & \\
0h & 5m & 15m & 30m & 1h & 2h & 6h \\
\hline
\text{p-AKT} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{AKT} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\beta\text{-actin} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\hline
\text{GnRH II 100nM} & & & & & & \\
\text{p-AKT} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{AKT} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\beta\text{-actin} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\end{array}
\]

\( (n=3, P<0.05) \)

B

\[
\begin{array}{ccc}
\text{GnRH I} & \text{---} & + & \text{---} & \text{---} & + & \text{---} \\
\text{GnRH II} & \text{---} & \text{---} & + & \text{---} & \text{---} & + \\
\text{LY294002} & \text{---} & \text{---} & \text{---} & + & + & + \\
\end{array}
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\begin{array}{ccc}
\text{TWIST} & \text{---} & \text{---} & \text{---} & + & \text{---} \\
\text{N-CADHERIN} & \text{---} & \text{---} & \text{---} & + & \text{---} \\
\beta\text{-actin} & \text{---} & \text{---} & \text{---} & + & \text{---} \\
\end{array}
\]

\( (n=3, P<0.05) \)
CHAPTER 5: GnRH REGulates MMP2 AND MMP9 EXPRESSION AND TROPHObLAST INVASION VIA PI3k/AKT- AND ERK1/2-MEDIATED RUNX2 EXPRESSION

5.1 Introduction

ECM components undergo dynamic changes in first trimester human pregnancy, with elevated expression of collagen types I, III, and V, as well as *de novo* expression of collagen IV (Aplin et al., 1988; Kisalus et al., 1987). Degrading such a complex ECM requires a combination of multiple members of the MMP family. Nearly all the MMP members are detected in the first-trimester human placenta (Weiss et al., 2007). MMP2 and MMP9 proteins are abundantly detected in the invading extravillous trophoblast (EVT) cells (Bai et al., 2005), and the expression of these two gelatinases in trophoblastic cells are highly related to cell invasiveness (Suman and Gupta, 2012). Hussein and colleagues reported a significant decrease in MMP-9 protein expression in preeclamptic placenta compared with the normal placenta (Omran et al., 2011; Shokry et al., 2009), which could be related to impaired trophoblast invasion in preeclampsia. Additionally, trophoblast MMP2 and MMP9 levels decline during the second and third trimesters, paralleling the time frame of reduced trophoblast invasion in late or term placentas (Shimonovitz et al., 1994).
The temporal and spatial expression of MMP-2 and MMP-9 in EVTs is regulated by multiple autocrine and paracrine factors. Numerous hormones, cytokines, and growth factors, such as human chorionic gonadotropin, epidermal growth factor, and interleukin-10, have been reported to either up- or down-regulate MMP-2 and MMP-9 in trophoblast cells (Dilly et al., 2010; Fluhr et al., 2008; Roth and Fisher, 1999). In addition to these, the GnRH-GnRHR system is present in first-trimester human placenta (Cheng et al., 2000; Chou et al., 2004; Lin et al., 1995), and GnRH has been shown to regulate MMP-2 and MMP-9 in human EVT cells (Chou et al., 2003c). GnRH appears to exert these effects via transactivation of epidermal growth factor receptor (Liu et al., 2009), however the downstream transcription factor(s) involved have not been studied.

Runt-related transcription factor 2 (RUNX2) is one of the three related transcription factors that control numerous biological functions in organ development, tissue differentiation, and cell fate (Komori, 2002, 2008). RUNX2 was first identified as a regulator of chondrocyte maturation and osteoblast differentiation (Prince et al., 2001). Interestingly, the effects of RUNX2 on chondrocyte and osteoblast differentiation were shown to be coupled with osteoblast cell migration (Fujita et al., 2004). More recently, RUNX2 has been extensively studied in cancer biology because of its important role in controlling cell migration and invasion (Pratap et al., 2006). In breast cancer cells, RUNX2 up-regulates MMP2 and MMP9 mRNA and protein expression as well as cell invasion (Pratap et al., 2005). We hypothesized that GnRH induces RUNX2 expression and regulates trophoblast invasion by inducing MMP-2 and MMP-9 expression.
5.2 Results

5.2.1 RUNX2, MMP2, and MMP9 are co-expressed in first-trimester human placenta cell column.

We first sought to detect RUNX2, MMP-2, and MMP-9 at sites of invasion in first-trimester human placenta. Abundant RUNX2 immunoreactivity was detected in the nucleus of both column EVT and cytotrophoblast cell populations in first-trimester human placenta. MMP-2 immunoreactivity was high in the distal end of the cell column approaching the invasive front, but not in the cytotrophoblast and syncytiotrophoblast. MMP-9 immunoreactivity was evident in the first-trimester cell column and was universally observed in the cytotrophoblast cell population (Fig. 5.1, A).

5.2.2 The differential expression of RUNX2 in trophoblastic cells is correlated with cell invasion

Having demonstrated the localization of RUNX2 in first-trimester placenta, we next examined the mRNA and protein levels of RUNX2 in primary EVTs and trophoblastic cell lines using semiquantitative PCR and Western blot analysis. Highly invasive primary EVT and HTR-8/SVneo cells display higher RUNX2 mRNA and protein level compare
to low invasive choriocarcinoma cell lines JAR, JEG-3 and BeWo (Fig. 5.1.B).

5.2.3 GnRH stimulates RUNX2 expression in HTR-8/SVneo cells

We used RT-qPCR and Western blot analysis to investigate the effects of GnRH on RUNX2 expression in HTR-8/SVneo cells. Treatment with 100 nM GnRH I or GnRH II significantly elevated RUNX2 mRNA levels at 24, 48, and 72 h compared with time-matched controls (Fig. 5.2.A). RUNX2 protein levels were also increased between 24 and 72 h after treatment with GnRH (Fig. 5.2.B). Concentration-response studies (1-100 nM GnRH) were also carried out in HTR-8/SVneo cells at the 24 h time-point, and only the 100 nM concentration was able to significantly increase RUNX2 mRNA and protein levels (Fig. 5.2.C and D).

5.2.4 Antide attenuates GnRH-induced RUNX2 expression

Antide was used to determine the role of GnRHR on GnRH-induced RUNX2 expression. Different concentrations of Antide (1, 10, and 100 nM) were added to the culture medium before a fixed concentration of GnRH (100 nM) was added. Pre-treatment with 100 nM Antide significantly reduced GnRH I- and GnRH II-induced RUNX2 mRNA and protein levels (Fig. 5.3.A and B).
5.2.5 AKT and ERK1/2 signaling mediates GnRH-induced RUNX2 expression

To examine the intracellular signaling mediating the effects of GnRH, we incubated HTR-8/SVneo cells with 100 nM GnRH for different amounts of time (5 min, 15 min, 30 min, 1 h, 2 h, and 6 h). Our results in Chapter 4 (Fig. 4.6 A) showed an increase in the phospho-AKT protein level from 5 min to 2 h in both the GnRH I and GnRH II treatment groups. In addition, we also observed a rapid elevation of ERK1/2 phosphorylation after GnRH treatment starting at 5 min and returning control levels by 30 min, followed by a second elevation from 2-6 h (Fig. 5.4.A). A PI3K inhibitor (LY294002) and a MEK inhibitor (PD98059) were used to investigate the roles of the AKT and ERK1/2 signaling in GnRH-induced RUNX2 expression. Pre-treatment for 1 h with either LY294002 or PD98059 attenuated both GnRH I- and GnRH II-induced RUNX2 protein production, (Fig. 5.4.B). Interestingly, basal RUNX2 protein levels were significantly decreased by treatment of either LY294002 or PD98059 alone.

5.2.6 RUNX2 regulates MMP-2 and MMP-9 expression and HTR-8/SVneo cell invasion

RUNX2 has been shown to regulate MMP-2 and MMP-9 expression in prostate cancer cells (Akech et al., 2010). Thus, to investigate the effects of RUNX2 on MMP-2
and MMP-9, transient siRNA transfection was used to down-regulate endogenous RUNX2 protein levels. Significant reductions in the mRNA levels of both MMP-2 and MMP-9 were observed following RUNX2 knockdown (Fig. 5.5.A and B). Likewise, depletion of RUNX2 suppressed the protein levels of pro-MMP-2 as well as pro- and active forms of MMP-9 (Fig. 5.5 C). Importantly, knockdown of endogenous RUNX2 attenuated overall Matrigel invasion of HTR-8/SVneo cells (Fig. 5.5.D).

To confirm the involvement of MMP-2 and MMP-9 in HTR-8/SVneo cell invasion, we performed transient knockdown of MMP-2 or MMP-9 prior to assaying Matrigel or collagen I mediated cell invasion. Significant reductions in invasive cell numbers were observed following depletion of MMP-2 or MMP-9 in both the Matrigel-coated (Fig. 5.E) or rat tail collagen I-coated transwell assays (Fig. 5.F).

5.2.7 RUNX2 mediates GnRH-induced MMP-2 and MMP-9 expression and HTR-8/SVneo cell invasion

Having demonstrated the involvement of RUNX2 in basal MMP2/9 expression and trophoblast invasion, we sought to test the role of RUNX2 in GnRH-induced cell invasion. We observed that GnRH-induced MMP-2 and MMP-9 protein levels were completely abolished after RUNX2 siRNA transfection in HTR-8/SVneo cells (Fig. 5.6.A). Moreover, treatment with RUNX2 siRNA significantly reduced GnRH-stimulated
Matrigel invasion of HTR-8/SVneo cells (Fig. 5.6.B).

5.2.8 MMP-2 and MMP-9 are involved in GnRH-induced HTR-8/SVneo cell invasion

Two distinct approaches were used to examine the involvement of MMP-2 and MMP-9 in GnRH-induced HTR-8/SVneo cell invasion. Co-transfection with MMP-2 and MMP-9 siRNA significantly inhibited GnRH-induced HTR-8/SVneo cell invasion (Fig. 5.6.C). In addition, pre-incubation with a MMP2/MMP9 inhibitor attenuated GnRH-induced HTR-8/SVneo cell invasion (Fig. 5.6.D).

5.3 Discussion

In our current study, we observed abundant immunoreactivity of the MMP-2 and MMP-9 proteins in the cell column region of the first-trimester human placenta specimens. In agreement with our finding, other groups also reported peak levels of MMP-2 and MMP-9 at 6-12 weeks of gestation (Staun-Ram et al., 2004), paralleling the higher expression of GnRH I and GnRH II in early gestation (Siler-Khodr and Khodr, 1978). Moreover, our previous finding has reported the presence of GnRHR in EVT cells isolated from the first-trimester human placenta (Cheng et al., 2000). Based on our
immunohistochemistry results, GnRHR, RUNX2 and MMP2/9 are jointly expressed in the distal region of cell column, remarking of acquisition of cell invasiveness. The temporospatial expression of GnRH-GnRHR, RUNX2, and gelatinases during early implantation may indicate the potential role of GnRH in regulating RUNX2- and gelatinase-mediated trophoblast function, especially invasive trophoblast commitment.

The expression of RUNX2 in cells is frequently associated with cell motility and invasiveness. For example, aberrantly increased RUNX2 expression was observed in metastatic breast cancer and prostate cancer cell lines, as well as in primary breast and prostate tumors, compared to less invasive cancer cell lines (Ferrari et al., 2012). Their data are also in accordance with our observations that greater RUNX2 expression was detected in the highly invasive primary EVT cells and HTR-8/SVneo cells compare to less-invasive JEG-3, JAR, and BeWo cells. Moreover, inhibition of RUNX2 in several carcinoma cells lead to a decrease of cell invasiveness (Niu et al., 2012; Wang et al., 2013), and we have observed similar phenomenon in invasive EVT cells.

The studies described in this chapter are the first to demonstrate stimulatory effects of GnRH on RUNX2 expression. GnRH has been reported to activate several noncanonical signaling pathways, such as the PI3K/AKT and MEK/ERK pathways, in various cell types (Hong et al., 2012; Ling Poon et al., 2011; Liu et al., 2002). Our findings are consistent with these reports and further demonstrate the importance of both AKT and ERK signaling in mediating the effects of GnRH on RUNX2 in HTR-8/SVneo cells. Both PI3K/AKT and MEK/ERK signaling have been reported to be essential for the
induction of RUNX2 expression by growth factors, mechanical sensation, or oxidative stress (Niger et al., 2012; Shi et al., 2011). Differently, some have reported that PI3K/AKT, but not MEK/ERK, signaling mediates the transcriptional induction of RUNX2 by IGF-1 in bone morphogenesis (Chen et al., 2012; Qiao et al., 2004). Based on our observations, in trophoblastic cells, both AKT and ERK signaling were involved, whereas AKT signaling exerted a stronger effect compared with the ERK pathway in regulating GnRH-induced RUNX2 expression. Differences between previous findings and our results are likely attributable to the diverse origins of the cell types in question and distinct mechanisms of ligand-induced signaling.

Our results demonstrate that knock down of RUNX2 decreases both MMP-2 and MMP-9 expression in trophoblastic cells. In agree with our finding, decreased MMP2/9 levels have been observed after the endogenous ablation of RUNX2 in prostate cancer cells, leading to a decrease in cancer cell invasion and bone metastasis (Akech et al., 2010). Moreover, RUNX2 has been shown to bind with multiple RUNX2 binding sites upstream (between 250 and 800 kb) of the MMP9 promoter region using ChIP assays (Pratap et al., 2005), showing that RUNX2 may regulate MMP9 gene expression via direct transcriptional regulation.

GnRH has also been reported to induce MMP2 and MMP9 expression in ovarian cancer cells, pituitary cells, and decidual stroma (Cheung et al., 2006; Chou et al., 2003b; Roelle et al., 2003). However, the underlining mechanisms are different. For example, previous findings indicate that GnRH could activate JNK phosphorylation, leading to an
increase of MMP2/9 expression in ovarian cancer (Cheung et al., 2006). Transactivation of EGFR has also been reported to be upstream of GnRH-induced MMP2/9 expression (Liu et al., 2009). However, in pituitary cells, a rapid increase of MMP2/9 (less in 5mins) was observed after GnRH administration, and was shown to mediate GnRH signal transduction to EGFR (Roelle et al., 2003). Our finding regarding the involvement of RUNX2 in GnRH-induced MMP2 and MMP9 expression has demonstrated a de novo mechanism for the regulation of gelatinases by GnRH. However, the role of transactivated EGFR in GnRH-induced RUNX2 expression is yet to be determined. In my study, both MMP2 and MMP9 were found to contribute to basal trophoblast invasiveness, and so far no evidence indicates their distinct mechanisms in regulating trophoblast invasion.

In summary, our studies show that GnRH, via its receptor, stimulates RUNX2 expression and is dependent on AKT and ERK1/2 phosphorylation. Increased levels of RUNX2 subsequently induce MMP2 and MMP9 expression and cell invasion of trophoblasts during placentation. These studies reveal, for the first time, the expression pattern of RUNX2 in the first-trimester human placenta and its functional impact in regulating trophoblast invasion.
Figure 5. 1 The expression of RUNX2, MMP2, and MMP9 in the first-trimester human placenta and trophoblastic cells.

Paraformaldehyde fixed and paraffin-embedded first trimester human placenta sections were deparaffinized and rehydrated by serial incubations in xylene and ethanol solution (see Chapter 2 section 2.4). Tissue samples were incubated overnight with primary antibody against RUNX2, MMP2, MMP9 or Ctrl IgG before secondary antibody incubation. DAB chromogen reagent was used for signal detection followed by nucleus counterstaining with hemotoxylene. The specimens were observed under a light microscope and all experiments were repeated three times. Immunohistochemistry staining of RUNX2, MMP2 and MMP9 in first trimester human placenta is shown in the micrographs (A) (100×, scale bar = 100 µM, micrograph in corner 400×, scale bar = 25 µM). The cell column region of first trimester human placenta is indicated by arrow.

Primary EVT cells, HTR-8/SVneo cells, choriocarcinoma JEG-3, JAR and BeWo cells were cultured and the cells were collected for semiquantitive RT-PCR or western blot analysis. The mRNA and protein levels of RUNX2 in trophoblastic cell lines and primary EVT cells are presented in micrograph (B). GAPDH was used as a reference gene for semiquantitive RT-PCR and all immunoblots are reprobed probed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. Each experiment was repeated three times with the same condition (n=3, P<0.01, Tukey test after one-way ANOVA).
(n=3, P<0.01)
Figure 5. 2 GnRH stimulates RUNX2 expression with different incubation periods and concentrations.

HTR-8/SVneo cells were incubated with 100nM of GnRH I or GnRH II. DNAse and RNAse water was used as the vehicle ctrl for GnRH. The cells were collected at different time point (0h, 12h, 24h, 48h and 72h) after treatment for mRNA extraction or SDS-PAGE. The mRNA and protein expression of target genes in these cells were analyzed by qRT-PCR and western blot, respectively. The immunolots were scanned and signals were quantified by Genetools software. RUNX2 mRNA levels and protein levels at different time point after GnRH treatment are presented in bargraphs (A) and (B), respectively. The mRNA level of RUNX2 each time point control is normalized to 1 in bargraph (A) and is presented as Ctrl. The protein level of RUNX2 in 0h is normalized to 1 in bargraph (B) and is presented as 0h. HTR-8/SVneo cells were incubated without GnRH or with GnRH in different concentration (1nM, 10nM and 100nM). The cells are collected for qRT-PCR or western blot at 48h after incubation. The mRNA and protein levels of RUNX2 after treatment with GnRH in different concentration are presented in the bargraphs (C) and (D), respectively. The mRNA and protein value of RUNX2 without GnRH treatment is normalized to 1 and is presented as Ctrl in bargaphs (C) and (D). Each experiment were repeated three time on independent occasions (n=3) and all data are presented as mean ± S.E.M.. Different letters in the bargraphs indicates significant differences (P<0.05, Tukey test after one-way ANOVA).
Figure 5. 3 The GnRH antagonist Antide attenuates GnRH-induced RUNX2 expression.

HTR-8/SVneo cells were pre-incubated with different concentrations (0, 1nM, 10nM and 100nM) of Antide 1h before treatment with 100nM of GnRH. DNAse and RNAse free water is used as the vehicle control of GnRH and Antide. The cells were collected at 48h after GnRH treatment for mRNA extraction or SDS-PAGE. The mRNA and protein expression of target genes in these cells were analyzed by qRT-PCR and western blot, respectively. All immunoblots were probed for β-actin to show relatively amounts of lysates loading in SDS-PAGE. The immunolots were scanned and signals were quantified by Genetools software. The mRNA and protein levels of RUNX2 after treatment with Antide in the presence of GnRH are shown in bargraphs (A) and (B), respectively. The values of RUNX2 mRNA and protein with GnRH treatment only are normalized to 1 and are presented as Ctrl in bargraphs (A) and (B). Three independent repeats were performed in each experiment and all data are presented as mean ± S.E.M.. Different letters in the bargraphs indicates significant differences (n=3, P<0.05, Tukey test after one-way ANOVA).
A

RUNX2 mRNA levels/GAPDH (fold change)

100nM
0
100nM
1nM
100nM
10nM
100nM
100nM
GnRH Antide

B

Antide 0 1nM 10nM 100nM

GnRH I
100nM
48h

RunX2

β-actin

GnRH II
100nM
48h

RunX2

β-actin

RUNX2 protein levels/β-actin (fold change)

100nM
0
100nM
1nM
100nM
10nM
100nM
100nM
GnRH Antide
Figure 5. GnRH stimulates RUNX2 expression via the PI3K/AKT and ERK1/2 signaling pathways.

HTR-8/SVneo cells were incubated with GnRH I and GnRH II and cells were collected in different time points (5m, 15m, 30m, 1h, 2h and 6h). DNAse and RNase free water were used as the vehicle control for GnRH. ERK1/2 and phosphor-ERK1/2 protein levels were detected using western blot and a representative immunoblot was shown in micrograph (A). HTR-8/SVneo cells were pre-incubated with DMSO, LY294002 or PD98095 for 1h before 100nM GnRH treatment. The whole cell lysates were collected at 48h after GnRH treatment. The protein in the whole cell lysates were separated using SDS-PAGE and a RUNX2 specific antibody was used to detect the expression of RUNX2 protein in the cell lysates. All immunoblots were reprobed for β-actin to show the amount of cell lysates loading in SDS-PAGE. Immunoblots were scanned and quantified using Genetool Software and relative RUNX2 protein levels in these cells were presented in the bargraph (B). The value of RUNX2 protein level in HTR-8/SVneo cells with DMSO only is normalized to 1 and presented as Ctrl in bargraph (B). All the experiments were repeated three times in independent occasions and all data are presented as mean ± S.E.M.. Different letters in the bargraphs indicates significant differences (n=3, P<0.05, Tukey test after one-way ANOVA).
Figure 5. Knockdown of RUNX2 reduces MMP2 and MMP9 expression and HTR-8/SVneo cell invasiveness.

HTR-8/SVneo cells were transfected with control siRNA or RUNX2 siRNA. The cells were either collected at 24h and 48h after transfection or used for Matrigel mediated transwell invasion assay (see Chapter 2 section 2.10). The mRNA levels of MMP2 and MMP9 mRNA levels were tested using Real-time PCR and are presented in the bargraphs (A) and (B), respectively. The expression levels of pro-MMP2, pro- and active-MMP9 protein were examined using western blot and are shown in the micrograph. The overall cell invasive capacity of HTR-8/SVneo after siRUNX2 transfection is presented in bargraph (D). Specific siRNAs against MMP2 and MMP9 were transfected into HTR-8/SVneo cells. Matrigel and collagen I mediated invasion assay (see Chapter 2 section 2.10) was performed using these cells. The cells invaded to the bottom of Boyden chambers were stained with hematoxylin quick-staining kit and were manually counted. Both Matrigel mediated (E) as well as collagen I mediated (F) cell invasiveness after knockdown of MMP2 and MMP9 is presented in the bargraphs. The values of mRNA, protein and invasiveness of HTR-8/SVneo cells with transfection reagent only are normalized to 1 and are presented in Vehicle. All the experiments were repeated three different times (n=3) and all data are presented as mean ± S.E.M.. Significant differences are indicated with different letters in the bargraphs (P<0.05, Tukey test after one-way ANOVA in (A), (B), (D), (E) and (F), Student-t test in (C)).
A

![Graph showing MMP-2 mRNA levels (fold change) at 24h and 48h for different treatments: Vehicle, Ctrl siRNA, siRunX2.](image)

B

![Graph showing MMP-9 mRNA levels (fold change) at 24h and 48h for different treatments: Vehicle, Ctrl siRNA, siRunX2.](image)

C

![Western blot images of Pro-MMP2, Pro-MMP9, Active-MMP9, and β-actin proteins at 48h for Ctrl siRNA and siRunX2 treatments.](image)

(n=3, P<0.05)
Figure 5. 6 Loss of function RUNX2 or MMP2/9 attenuates GnRH-induced HTR-8/SVneo cell invasion.

HTR-8/SVneo cells were incubated with 100nM GnRH since 24h after RUNX2 or MMP2/9 siRNA transfection. The cells were either collected 48h after GnRH treatment or seeded for invasion assay 24h after GnRH treatment. The whole cell lysates were loaded in SDS-PAGE and western blot was used to test the MMP2/9 protein expression in these cells. Representative pro-MMP-2, pro- and active-MMP-9 protein expressions are shown in the chemiluminograms (A). The data are presented as mean in micrograph (A). The cell invasiveness of HTR-8/SVneo cells after RUNX2 knockdown with the presence of GnRH is presented in the bargraph (B). GnRH induced cell invasiveness after MMP2/9 siRNA transfection was examined and presented in the bar graph (C). MMP2/9 inhibitor was added into HTR-8/SVneo culture medium 24h after GnRH treatment. DMSO was used as the vehicle control of MMP2/9 inhibitor. The cells were placed into boyden chamber for invasion assay 1h after MMP2/9 inhibitor incubation. The cell invasiveness of HTR-8/SVneo cells after co-incubation of MMP2/9 inhibitors and GnRH is presented in bargraph (D). The values in ctrl siRNA transfection group or DMSO only group were normalized to 1. Each experiment were repeated three time on independent occasions (n=3) and data in bargraph (B), (C) and (D) are presented as mean ± S.E.M.. Different letters in the bargraphs indicates significant differences (P<0.05, Tukey test after one-way ANOVA).
CHAPTER 6: GnRH REGULATES CADHERIN-11 EXPRESSION VIA ACTIVATION OF C-FOS AND C-JUN IN TROPGOBLASTIC CELLS

6.1 Introduction

In first trimester human placenta, both native GnRH I and secondary form GnRH II exists in EVT cell population (Chou et al., 2004; Khodr and Siler-Khodr, 1980). The presence of GnRHR in first trimester EVT cells has also been demonstrated in previous reports (Cheng et al., 2000; Lin et al., 1995) and my immunohistochemistry study in Chapter III. Our previous reports have demonstrated the pro-invasive role of GnRH in regulating trophoblastic cell invasion via modulating proteolytic balance such as MMP/TIMP and uPA/PAI system in primary EVT cells (Chou et al., 2003a; Chou et al., 2003c). Moreover, the results presented in Chapter IV have revealed a de novo pro-invasive mechanism of GnRH by mediating N-cadherin upregulation in EVT cells. This evidence suggests that GnRH may regulate trophoblast invasion by promoting cellular mesenchymal characteristics.

Cadherin-11 (OB-cadherin), firstly found in neural tube during early development, is located primarily in mesenchymal tissues and is often associated with cellular mesenchymal phenotypes (Hoffmann and Balling, 1995; Kimura et al., 1995; Simonneau et al., 1995). After been demonstrated to play key roles in early development, cadherin-11
has also been detected in both maternal and fetal side of human placenta (MacCalman et al., 1996). Abundant Cadherin-11 immunoreactivity has been observed in both multinucleate syncytiotrophoblast and invasive EVT cell populations (MacCalman et al., 1996). Induction of cell fusion has been observed after exogenous overexpression of cadherin-11 in JEG-3 cells, suggesting that cadherin-11 may play critical roles in regulating trophoblast fusion (Getsios and MacCalman, 2003). However, the function of cadherin-11 in invasive EVT cells has yet been investigated, although expression of cadherin-11 has long been found in these cells.

Multiple transcription factors required in early development such as activator protein-1 (AP-1) has been reported to be activated by GnRH in pituitary cells, showing their critical roles in mediating GnRH induced downstream gene regulation (Binder et al., 2012; Liu et al., 2002). Interestingly, as the major component of AP-1 transcription factor, both c-FOS and c-JUN are detected in invasive EVT cell population (Bamberger et al., 2004; Briese et al., 2005). Moreover, alternative expressions of c-FOS and c-JUN has been observed in preeclamptic patients compared to those in normal control patients, suggesting that these transcription factors may be essential in controlling early placenta development (Marzioni et al., 2010). However, to date, the role of GnRH in regulating c-FOS and c-JUN in trophoblastic cells is still unknown, and the involvement of cadherin-11 in GnRH induced mesenchymal cadherin remodeling is also yet to be determined.
6.2 Results

6.2.1 Phosphorylated c-FOS and c-JUN are localized in EVT nucleus in first-trimester human placenta

In order to determine the cellular localization of AP-1 component in first-trimester human placenta, specific antibodies against p-c-FOS and p-c-JUN were performed in immunofluorescence study. P-c-FOS could not be detected in the cell nucleus of either villous cytotrophoblast or syncytiotrophoblast, whereas abundant p-c-FOS immunofluorescence signals could be observed in the EVT cell nucleus at cell column region (Fig.6.1 A). Interestingly, elevated p-c-FOS could be detected in the distal end of cell column compared to basal lamina, correlating with the expression of EVT cell marker HLA-G. P-c-JUN could also be detected in column EVT cell nucleus. However, the signal of p-c-JUN is not restrained in EVT cells, but could also be detected in mononucleate cytotrophoblastic cells (Fig.6.1 A).

6.2.2 GnRH stimulates c-FOS and c-JUN expression and phosphorylation

Having determined the localization of c-FOS and c-JUN in first-trimester EVT cells, we then use immortalized HTR-8/SVneo cells to study the capability of GnRH to
regulate the expression and activation of these transcription factors. Rapid elevation of both FOS and JUN mRNA levels was observed within 3h after GnRH treatment according to our qRT-PCR results (Fig.6.2.A). The mRNA level of FOS returned to normal after 12h, whereas the expression level of JUN transcriptome remain elevated till 12h after GnRH administration (Fig.6.2.A). Our western blot results showed that phosphorylation of c-FOS and c-JUN was also significantly increased after treatment of HTR-8/SVneo cells with GnRH (Fig. 6.2.B). Additionally, both c-FOS and c-JUN proteins were accumulated in HTR-8/SVneo cell nucleus at 1h after GnRH administration (Fig.6.2.C).

6.2.3 GnRH increases cadherin-11 expression in HTR-8/SVneo cells

Having demonstrated that GnRH potentiated c-FOS and c-JUN expression, we then investigated the role of GnRH in regulating cadheirin-11 expression. A significant increase of cadherin-11 mRNA and protein levels was observed at 12h after administration with 100nM of both GnRH I and GnRH II, with prolonged stimulatory effect till 48h (Fig. 6.3.A and B). After treatment with different concentration of GnRH (0, 1nM, 10nM and 100nM), the mRNA and protein expression of cadherin-11 in HTR-8/SVneo cells was significantly elevated only at higher concentrations (10nM and 100nM), but not at lower concentration (Fig. 6.3.C and D).
To investigate the involvement of GnRHR in GnRH-induced cadherin-11 expression, GnRH antagonist Antide was pre-incubated with HTR-8/SVneo cells before GnRH treatment. Our western blot results indicated that pre-treatment with Antide successfully abolished GnRH induced cadherin-11 expression (Fig. 6.3.E).

6.2.4 Transcription factor c-FOS and c-JUN regulates cadherin-11 expression and trophoblast invasion

As shown in Fig. 6.4.A and B, inhibition of c-FOS and c-JUN expression with transient siRNA transfection successfully attenuated endogenous cadherin-11 mRNA and protein expression in HTR-8/SVneo cells, respectively. Moreover, knockdown of c-FOS and c-JUN yielded significant decreased overall cell invasiveness in HTR-8/SVneo cells (Fig.6.4.C). Similarly, the endogenous down-regulation of cadherin-11 also attenuated the basal HTR-8/SVneo cell invasion (Fig. 6.4. D).

6.2.5 GnRH-induced cadherin-11 expression and trophoblast invasion is mediated by c-FOS and c-JUN

Having determined that both c-FOS and c-JUN could regulate basal cadherin-11 expression, we further investigated the role of c-FOS and c-JUN in GnRH-mediated
cadherin-11 and trophoblast invasion. As shown in Fig. 6.5.A, GnRH-induced cadherin-11 expression was attenuated by transfection of FOS and JUN specific siRNA in HTR-8/SVneo cells. Meanwhile, significantly attenuated HTR-8/SVneo cell invasion with GnRH treatment was observed after FOS, JUN and cadherin-11 siRNA transfection (Fig.6.5.B). Interestingly, only partial attenuation of GnRH-induced cell invasion was detected after FOS, JUN and cadherin-11 siRNA transfection in HTR-8/SVneo cells.

6.3 Discussion

In our study, we have observed that both the mRNA and protein levels of c-FOS and c-JUN are increased after administration of GnRH in immortalized HTR-8/SVneo cells. In agree with our finding, previous studies have demonstrated that GnRH is capable to activate c-FOS and c-JUN in pituitary cells (Binder et al., 2012; Liu et al., 2002). However, distinct gene activation pattern has been observed in trophoblastic and pituitary cells. For example, rapid c-FOS and c-JUN mRNA accumulation has been observed within an hour after GnRH administration in pituitary cells, whereas in our experimental condition, peak effect appears at 3h after treatment. Moreover, more predominant effect of c-FOS and c-JUN mRNA activation has been detected (more than 10 fold higher) in pituitary cells compared to that in trophoblasts. The presence of this distinct phenomenon may due to the differential expression levels of GnRHR in pituitary and placenta cells. A 10 fold higher GnRHR mRNA level has been detected in mouse pituitary compared to
mouse ovary (Torrealday et al., 2013). Based on my result in Chapter III, a further 10 fold higher GnRHR protein level has been observed in human primary granulosa cells compared to HTR-8/SVneo cells. A relatively lower GnRHR level in trophoblastic cells may lead to slower response of target gene regulation induced by ligand.

Transcriptional activation of c-FOS and c-JUN requires nuclear translocation, often accompanied with an requisition of transcription factor phosphorylation (Nardozzi et al., 2010). Both c-FOS and c-JUN are relatively unstable and undergo rapid nuclear turnover process (Vesely et al., 2009). Here we detected the presence of phospho-c-FOS and phospho-c-JUN at the EVT cell nucleus of first trimester human placenta cell column, showing that activated AP-1 component may potentiate trophoblastic function. Phosphorylation status of c-FOS and c-JUN is regulated by numerous cell signaling (Vesely et al., 2009). For example, JNK signaling has been reported to conjugate with phosphorylation of c-JUN in response of cell stress (Leppa and Bohmann, 1999). Moreover, further studies have demonstrated that c-JUN is capable to be phosphorylated by ERK and p38 MAPK (Karin, 1995; Tanos et al., 2005). Our previous finding has demonstrated that GnRH could potentiate c-JUN phosphorylation via JNK signaling in primary EVT cells (Liu et al., 2009), whereas involvement of additional signalings in GnRH mediated AP-1 activation still need further investigated.

We are the first to report that GnRH could regulate cadherin-11 expression in trophoblastic cells. Limited studies have analyzed the transcriptional regulation of cadherin-11 expression. Inhibition of GSK3β has been reported to suppress cadherin-11
expression, whereas GSK3β is not required for RNA polymerize II recruitment to cadherin-11 promoter (Farina et al., 2009). In my study, knockdown of either c-FOS or c-JUN could inhibit both mRNA and protein expression of cadherin-11. However, the binding capability of c-FOS and c-JUN to cadherin-11 promoter still need to be further investigated.

Cadherin-11 has been reported to associate with tumor progression and invasion in breast tumor and prostate cancer (Huang et al., 2010; Li et al., 2011). Contrarily, cadherin-11 has also been regarded as a tumor suppressor in esophageal carcinomas, likely by inducing tumor cell apoptosis and modulating cell stemness. In my invasive trophoblastic cell model, knockdown of cadherin-11 attenuates both basal and GnRH induced trophoblast invasion, which correlates to our previous finding of abundant cadherin-11 expression in high invasive EVT cells.

In conclusion, our findings have demonstrated the pro-invasive roles of transcription factor c-FOS, c-JUN and mesenchymal adhesion molecule cadherin-11 in regulating trophoblast invasion. We have also suggested that GnRH regulates trophoblast invasion via a de novo pathway by inducing c-FOS/c-JUN mediated cadherin-11 expression. These findings provide additional understanding towards the potential paracrine and autocrine function of GnRH-GnRHR system in early implantation and plancentation.
Figure 6. Immunolocalization of phosphorylated transcription factors c-FOS and c-JUN in first-trimester human placenta villi.

First-trimester placenta samples were embedded in OCT and were rapidly frozen in liquid nitrogen before sectioning. Placenta sections (8µM) were fixed in cold acetone before staining. Specific rabbit p-c-FOS, p-c-JUN and mouse HLA-G antibodies were used for target molecule detection before anti-rabbit Alexa Fluo 594 or anti-mouse Alexa Fluo 488 secondary antibodies conjugation. DAPI was used for nucleus labeling. The sections were observed under a confocal microscope. The localization of phosphorylated c-FOS and phosphorylated c-JUN in first trimester human placenta were presented in the micrographs (A) and (B). The pictures are representative results from three independent experiments and the micrographs are presented in 100× magnification.
Figure 6. 2 GnRH induces expression and phosphorylation of c-FOS and c-JUN, and activates their nucleus accumulation.

HTR-8/SVneo cells were treated 100nM GnRH I and GnRH II. The cells were collected at different time points (1h, 3h, 6h and 12h) for mRNA isolation. The mRNA levels of FOS and JUN in these cells were tested using qRT-PCR and are presented in the bargraph (A). The values of HTR-8/SVneo cells in the time GnRH added were normalized to 1 and presented as 0h. All data are presented as the mean ± S.E.M. in the bargraph (A). Whole cell lysates of HTR-8/SVneo cells after GnRH treatment were collected in different time points (15m, 30m and 1h) and were loaded in SDS-PAGE. Representative immunoblots of p-c-FOS and p-c-JUN were shown in chemiluminogram (B). Total c-FOS and c-JUN were used as control. The values of HTR-8/SVneo cells in the time GnRH added were normalized to 1 and presented as 0. The numbers under each band shows the mean value in microgram (B). All data were derived from three independent experiments and different letters in the bargraphs represents significant differences (P<0.05, Tukey test after one-way ANOVA in (A) and (B)). HTR-8/SVneo cells were seeded on coverslips 24h ahead and GnRH was added 1h before fixation in cold methanol. The cells were immune-stained using specific rabbit c-FOS and c-JUN antibodies. Mouse α-tubulin antibody was used to label microtubule in cytoplasm. Cells were observed under a fluorescence microscope. The cellular localization of c-FOS and c-JUN after GnRH treatment was shown in representative pictures (C).
Figure 6. 3 GnRH induces cadherin-11 expression in HTR-8/SVneo cells.

HTR-8/SVneo cells were incubated either with 100nM of GnRH for different time (0h, 12h, 24h, 48h or 72h), or with different concentrations (0nM, 1nM, 10nM, or 100nM) of GnRH for 24h. The cells were collected for mRNA and protein extraction. QRT-PCR and western blot was used to test the mRNA and protein level of cadherin-11 in these cells. The mRNA and protein levels of cadherin-11 in different time points after GnRH treatment are presented in bargraphs (A) and (B), respectively. Control of each time point is normalized to 1 and is presented as Ctrl in bargraph (A). Cadherin-11 mRNA and protein levels under different concentration of GnRH administration are shown in bargraphs (C) and (D), respectively. The values without GnRH treatment were normalized to 1 in bargraphs (B), (C) and (D). Antide was added into HTR-8/SVneo cell culture medium 1h before 100nM GnRH treatment. DNase and RNAse free water was used as the vehicle control for GnRH and Antide. The cells were collected for western blot analysis at 24h after GnRH treatment. Quantitative cadherin-11 protein levels after co-treatment Antide and GnRH incubation are shown in bargraph (E). All data above were generated by statistically analysis of three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA).
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**Graphical Representation:**

- **Y-axis:** CAD-11 protein levels/tubulin (fold change)
- **X-axis:** Vehicle, Antide
- **Legend:**
  - Ctrl
  - GnRH I 100nM
  - GnRH II 100nM

**Annotations:**

- a: Significant difference from Ctrl
- b: Significant difference from GnRH I 100nM

**Note:**

- Each bar represents the fold change in protein levels compared to the control (Ctrl).
- Significant differences are indicated by letters (a, b).
- The images show representative Western blots for CAD-11 and α-tubulin.
Figure 6. 4 Transcription factors c-FOS and c-JUN regulate basal cadherin-11 expression and cell invasion in HTR-8/SVneo cells.

HTR-8/SVneo cells were transfected with control siRNA or specific siRNAs against c-FOS, c-JUN or cadherin-11. The cells incubated with transfection reagent only were used as vehicle control. After 48h, cells were collected for mRNA/protein extraction. The mRNA and protein levels of cadherin-11 after siRNA transfection were examined with qRT-PCR and western blot, respectively. All immunoblots were reprobed for α-tubulin to show the relative amount of lysates loaded in SDS-PAGE. The mRNA and protein levels of cadherin-11 after FOS/JUN siRNA transfection were presented in bargraphs (A) and (B), respectively. At 48h after knockdown of FOS/JUN and cadherin-11 in HTR-8/SVneo cells, the cells were trypsinized for Matrigel-mediated transwell invasion assay. The cells were seeded in the Boyden chamber and were incubated in 37°C for 24h before fixation in cold methanol. The invaded cells were stained using hemotoxylin and manually counted. The HTR-8/SVneo cell invasiveness after knockdown of c-FOS/c-JUN and cadherin-11 was presented in (C) and (D), respectively. The values in control siRNA group are normalized to 1 and are shown as Ctrl siRNA. All data above were generated by statistically analysis of three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA in (A), (B), and (C), Student-t test in (D)).
Figure 6. 5 Transcription factors c-FOS and c-JUN regulate GnRH induced cadherin-11 expression and cell invasion in HTR-8/SVneo cells.

HTR-8/SVneo cells were transfected with control siRNA or specific siRNAs against c-FOS, c-JUN or cadherin-11. The cells were treated with 100nM GnRH at 48h after transfection. DNAse and RNAse free water was used as the vehicle control of GnRH. The cells were collected for mRNA extraction at 24h after GnRH treatment. The mRNA levels of cadherin-11 in these cells were tested using qRT-PCR and were shown in the bargraph (A). At 24 after GnRH treatment of the transient transfected HTR-8/SVneo cells, the cells were trypsinated for Matrigel-mediated transwell invasion assay. The cells were seeded in the Boyden chamber and were incubated in 37°C for 24h before fixation in cold methanol. The invaded cells were stained using hemotoxylin and manually counted. The cell invasiveness of these transient transfected HTR-8/SVneo cells after GnRH treatment was presented in bargraph (B). The values in control siRNA group are normalized to 1 and are shown as Ctrl siRNA. All data were generated by statistically analysis of three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraphs (n=3, Tukey test after one-way ANOVA).
CHAPTER 7: GnRH REGULATES TROPHOBLAST VASCULAR REMODELING CAPABILITY VIA MODULATION OF MESENCHYMAL CADHERIN AND GELATINASE FAMILY OF MMPs EXPRESSION

7.1 Introduction

The increasing requirement of oxygen and nutrition from the growing fetus during early pregnancy imposes a heavy burden to the maternal body. To satisfy this rapid expanding demand from the fetus, remarkable changes occur in the growing placenta and uterus during pregnancy. Beginning at the first few weeks of pregnancy, winding low-flow and high-resistance endometrial spiral arteries are substituted by “horn-shaped” high-flow and low-resistance blood vessels (Brosens et al., 1967). Impaired spiral artery remodeling and inadequate reconstruction of the maternal endometrial vascular system may lead to severe consequences, such as preeclampsia and intrauterine growth restriction, in the placenta (Khong et al., 1986). The failure of de novo placenta adaptation may further cause insufficient maternal-fetal perfusion, leading to placenta structure damage, restriction of material supplements and hypertension (Gilbert et al., 2008; Roberts et al., 1989).

The process of endometrial vascular remodeling in early pregnancy requires synergetic cellular behaviors among vascular smooth muscle cells, endothelial cells,
EVTs, and maternal immune cells (e.g., dNKs). The vascular remodeling process is divided into several stages. A trophoblast-independent process with leukocyte vascular infiltration appears as the first stage during vascular remodeling, and EVT-induced removal of vascular smooth muscle cells and endothelial cells constitutes the second stage. EVT replacement of endothelial cells and extracellular matrix deposition are considered the final stage (Cartwright et al., 2010). The presence of endovascular EVT is traced as early as 8 weeks (Pijnenborg et al., 1980), and deep invasive interstitial EVT is also observed from the first trimester to midgestation (Pijnenborg et al., 1981). The coordination between interstitial and endovascular EVTs is essential and necessary for the reconstruction of the vascular wall. Structural changes in the vascular smooth muscle layer and endothelial cells are followed by interstitial EVT invasion (Pijnenborg et al., 1983). Evidence has shown that the presence of the interstitial EVT surrounding the vascular walls is the prerequisite for vascular structure disruption (Kam et al., 1999). Additionally, vascular EVTs acquire endothelial characteristics and express endothelial markers such as VE-cadherin vascular endothelial adhesion molecule-1 (VEAM-1) and α4 integrin (Zhou et al., 1997). EVT directly binds to vascular endothelial cells with VEAM-1 and the α4 integrin molecule pair in vitro (Zhou et al., 1997).

Trophoblast invasion has been known to be regulated by growth factors, cytokines, adhesion molecules, MMPs, and oxygen tension. Knowledge regarding the molecular mechanisms underlying endometrial vascular remodeling is still quite limited. Evidence has shown that during spiral artery remodeling, the appearance of certain angiogenic
growth factors shares certain similarities with angiogenesis (Geva et al., 2002), suggesting that a similar molecular mechanism may be involved in both processes.

Cell adhesion molecules and MMPs have been reported to regulate angiogenesis and vasculogenesis in embryo development and cancer progression (Blaschuk and Rowlands, 2000; Giannopoulou et al., 2008). For example, VE-cadherin, an endothelial cell marker, directly mediates endothelial cell adhesion, vascular permeability, and angiogenesis (Vestweber, 2008). N-cadherin is also expressed in endothelial cells and has been reported to regulate vascular morphogenesis together with VE-cadherin (Luo and Radice, 2005). In carcinomas, N-cadherin is also associated with tumor angiogenesis and poor prognosis (Nakashima et al., 2003). Cadherin-11 in fibroblasts mediates VEGF-D production, likely by contributing to neighboring vascular development (Orlandini and Oliviero, 2001). Both MMP-2 and MMP-9 are secreted in endothelial cells, and evidence has linked the expression of gelatinases with cell angiogenic behavior (Stetler-Stevenson, 1999). N-cadherin, cadherin-11, MMP2, and MMP9 are all predominately expressed in the EVT cell population during endovascular invasion, and GnRH modulates the expression of these molecules. Therefore, the purpose of my study was to investigate the regulatory effect of GnRH in EVT vascular morphogenesis and to elucidate the molecular mechanism that is involved in this process.
7.2 Results

7.2.1 GnRH stimulates trophoblast tube formation

To investigate the effect of GnRH on trophoblast tube formation capacity, the HTR-8/SVneo cells were pre-treated with 100 nM of either GnRH I or GnRH II for 24 h, and overall tube formation was analyzed using the Matrigel-based tube formation assay with or without the presence of GnRH. Increased overall capillary-like network coverage was observed with the presence of 100 nM GnRH compared with the vehicle control (Fig. 7.1, A). Three different angiogenic parameters, namely, the total branch point, total network number, and total tube length, were measured to quantify the tube formation capacity under the influence of GnRH treatment. The GnRH I treatment significantly increased the branch point, network number, and tube length in HTR-8/SVneo cells by 41%, 51%, and 87%, respectively (Fig. 7.1, B, C, and D). Similarly, all three angiogenic parameters were elevated by pre-incubation of GnRH II by 45%, 58%, and 93%, respectively (Fig. 7.1, B, C, and D).
7.2.2 Antide abolishes GnRH-induced trophoblast tube formation

The presence of GnRHR in the HTR-8/SVneo cells was studied in Chapter III, and the stimulatory effect of GnRH on cell behavior changes was considered to involve the interaction between GnRH and its receptor. To investigate the involvement of GnRHR in GnRH-regulated trophoblast tube formation, a GnRHR antagonist Antide was pre-incubated with the HTR-8/SVneo cells before GnRH treatment. As shown in Fig. 7.2, the HTR-8/SVneo cells formed an organized network on Matrigel and this effect was stimulated by both GnRH I and GnRH II. Pre-incubation of Antide did not affect basal capillary-like network formation but abolished GnRH-induced tube formation (Fig. 7.2.A). As shown in the bar graph, compared with GnRH alone, the total tube length was decreased by the co-incubation with Antide and GnRH (Fig. 7.2.B).

7.2.3 GnRH stimulates N-cadherin, cadherin-11, MMP-2, and MMP-9 expression in the HTR-8/SVneo cells

The pro-invasive effect of GnRH on trophoblastic cells was demonstrated in Chapters III and IV, which involves reprogramming of the cell adhesion molecules and proteolytic machinery. To further confirm the stimulatory effect of GnRH on the expression of mesenchymal cadherin and the gelatinase family of MMPs, I then
examined the mRNA and protein levels of N-cadherin, cadherin-11, MMP2, and MMP9 in the presence of 100 nM GnRH. Under this experimental condition, I observed a significant increase in N-cadherin, cadherin-11, MMP2, and MMP9 mRNA levels 24 h after GnRH treatment (Fig. 7.3.A). Moreover, elevated protein levels of all four target molecules were detected at 48 h after the pre-incubation of GnRH by Western blot analysis (Fig. 7.3, B).

**7.2.4 Endogenous N-cadherin, cadherin-11, MMP2, and MMP9 regulate trophoblast tube formation**

Cellular angiogenesis and vasculogenesis behaviors in multiple physiological and pathological conditions are considered to involve cell adhesion and proteolysis (Blaschuk and Rowlands, 2000; Giannopoulou et al., 2008). To investigate the potential roles of endogenous mesenchymal cadherins and gelatinases in regulating trophoblast tube formation, I then tested the effect of N-cadherin, cadherin-11, MMP2, and MMP9 siRNAs in the tube formation assay. As shown in Fig. 7.4 A, the knockdown of N-cadherin and cadherin-11 interrupted the formation of mass networks in the HTR-8/SVneo cells. The quantification results also showed that a significantly decreased total tube length was observed in the HTR-8/SVneo cells after knockdown of N-cadherin and cadherin-11 (Fig. 7.4 B). Moreover, functionally blocking N-cadherin by an
N-cadherin-perturbing antibody significantly attenuated HTR-8/SVneo tube formation (Fig. 7.4.C). Similarly, the down-regulation of endogenous MMP2 and MMP9 also inhibited the organization of capillary-like networks and attenuated the overall tube length in the HTR-8/SVneo cells (Fig. 7.5.A and B). In my studies in Chapter VI, I investigated the regulatory effect of RUNX2 on endogenous MMP2 and MMP9 expression in trophoblastic cells. The knockdown of endogenous RUNX2 in the HTR-8/SVneo cells also interrupted tube formation (Fig. 7.5.A and B). Additionally, the chemical inhibitor of MMP2 and/or MMP9 strongly hindered trophoblast tube formation (Fig. 7.5.C).

7.2.5 The down-regulation of N-cadherin, cadherin-11, MMP2, and MMP9 attenuates GnRH-induced trophoblast tube formation

To investigate the involvement of N-cadherin, cadherin-11, MMP-2, and MMP-9 in GnRH-mediated trophoblast tube formation, the siRNAs against these four genes were transfected into the HTR-8/SVneo cells 24 h before 100 nM GnRH I or GnRH II was added to the culture medium. The cells were trypsinized 24 h after GnRH treatment and were plated on top of the Matrigel overnight before observation. As shown in the bar graph of Fig. 7.6.A, the GnRH-stimulated increase in the total tube length was attenuated by transfection of siRNA against N-cadherin or cadherin-11. Similarly, MMP-2 and
MMP-9-specific siRNAs also inhibited GnRH-stimulated tube growth (Fig. 7.6.B). Interestingly, the knockdown of N-cadherin, cadherin-11, MMP2, or MMP9 alone partially attenuated but did not completely abolish the GnRH-induced capillary-like network organization.

7.3 Discussion

Vascular remodeling during early pregnancy plays crucial roles in embryo implantation and placenta development (Cartwright et al., 2010). The endothelial cell mimicry of EVTs, together with their invasive potential, makes these cells functionally capable of reconstructing maternal tissues from fetal origin (Harris, 2010). The functional and structural integrity of ECM components and cell adhesion molecules between trophoblast and endothelial cells directly influence vascular organization, endothelial cell fate, and the reconstruction of de novo vessels by trophoblasts (Whitley and Cartwright, 2010). Distinct molecular mechanisms have been demonstrated regarding the regulatory effect of GnRH on ECM degradation and cadherin reprogramming in previous chapters of my thesis. In the present chapter, for the first time, I demonstrate the role of GnRH on trophoblastic pro-angiogenic effects by employing an in vitro tube formation assay.

Limited studies have analyzed the role of GnRH in regulating cell angiogenesis. However, the modulation of angiogenesis that is related to chemokine secretion has been reported in an immortalized EVT cell line after GnRH treatment (Cavanagh et al., 2009).
Cavanagh and colleagues detected elevated mRNA expression of four different angiogenic-related chemokines, CXCL2, CXCL3, CXCL6, and CXCL8, after incubation of the HTR-8/SVneo cells with a synthetic GnRH I analog (buserelin) or native GnRH II. Additional experiments also revealed that accumulated CXCL8 appeared in the HTR-8/SVneo cell culture medium after GnRH treatment. This evidence has demonstrated that GnRH may induce the pro-angiogenesis effect of EVT by indirectly stimulating angiogenesis-related chemokines. Cavanagh and colleagues detected significantly elevated CXCL8 secretion in the conditioned medium at 12 h after GnRH treatment; however, the increased tube formation capability was already observed after the overnight incubation (12 h) of GnRH in the HTR-8/SVneo cells in my experiment. This observation indicates that molecules that potentiate the direct regulation of angiogenesis also participate in GnRH-stimulated trophoblast tube formation.

We are the first group to observe suppressed capillary-like network formation in the HTR-8/SVneo cells after the endogenous inhibition of N-cadherin and cadherin-11. The knockdown of N-cadherin in prostate cancer cells has been reported to lead to decreased human microvascular endothelial cell (HMEC) tube formation in vitro, likely involving the modulation of pro-angiogenic growth factors such as monocyte chemoattractant protein-1 secretion (Nalla et al., 2011). In this study, the endogenous down-regulation of N-cadherin was also reported to inhibit endothelial adhesion of the cancer cells, which share large similarity to trophoblast cells during vascular invasion. In my study, an N-cadherin functional perturbing antibody (Clone GC-4) successfully inhibited the
HTR-8/SVneo cell capillary-like network organization; however, no effect was reported in human umbilical vein endothelial cells (HUVECs) using the same antibody (Bach et al., 1998). Dominant VE-cadherin may potentially compensate for N-cadherin loss in HUVECs.

Various studies revealed the relationship between MMPs and angiogenesis, especially in the carcinogenesis process (Jackson, 2002; Rundhaug, 2005). In my experiment, the knockdown of MMP2 and/or MMP9 effectively inhibited Matrigel-mediated HTR-8/SVneo cell tube formation. The underlying mechanism(s) of their angiogenic regulatory role has been studied in endothelial cells. The endogenous suppression of MMP2 caused ablated endothelial cell tube formation, likely through the disrupted integrin αvβ3-CXCL-12 interaction (Maddirela et al., 2013). Interestingly, integrin αvβ3 has been reported to be expressed in EVT cells and likely to contribute to trophoblast invasion (Kabir-Salmani et al., 2003). MMP-2-αvβ3 signaling in EVTs may also contribute to trophoblastic angiogenic behavior. Similar to our observation, significantly decreased capillary-like network formation has also been shown in endothelial cells after the down-regulation of MMP9 (Jadhav et al., 2004). According to my data, a more dominant role of MMP9 in regulating HTR-8/SVneo cell angiogenesis has been observed compared with MMP2, which may be due to significantly higher MMP9 expression (10-fold higher at the mRNA level) in HTR-8/SVneo cells compared with MMP2 expression (Suman et al., 2012).
Taken together, the temporospatial expression of GnRH I and GnRH II during the first trimester not only provides the required EVT invasiveness but also potentiates the modulation of trophoblast vascular remodeling by reprogramming gelatinases and mesenchymal cadherins. Our current finding warrants further investigation into the role of GnRH in regulating trophoblast function.
Figure 7. 1 GnRH stimulates HTR-8/SVneo cell tube formation.

HTR-8/SVneo cells were pre-treated with 100nM GnRH I and GnRH II for 24h. DNAse and RNAse free water was used as vehicle control for GnRH. Matrigel induced tube-formation assay was performed using these cells (see Chapter 2 section 2.11). Undiluted Matrigel basement was placed in 96 well plates for 1h to form a gelatinized basement. The HTR-8/SVneo cells were seeded into the plate at a concentration of 2×10⁴ cells/well. The cells were incubated in 37°C for 24h. Capillary-like networks after GnRH treatment were observed under a light microscope and were presented in micrograph (A). Images covering the entire tube network in each well were obtained. Total branching point (B), total network number (C) and total tube length (D) were quantified using Northern Eclipse image analysing software and were presented in the following bargraphs. The relative value of these three parameters in control group were normalized to 1 and presented as Vehicle Ctrl. All data were derived from three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA).
**Figure 7. 2 Antide abolishes GnRH induced HTR-8/SVneo cell tube formation.**

HTR-8/SVneo cells were pre-treated with 100nM Antide for 1h before 100nM GnRH I and GnRH II treatment. DNAse and RNAse free water was used as vehicle control for GnRH and Antide. Undiluted Matrigel basement was placed in 96 well plates for 1h to form a gelatinized basement. The HTR-8/SVneo cells were seeded into the plate at a concentration of $2 \times 10^4$ cells/well. The cells were incubated in 37°C for 24h. Capillary-like networks after co-treatment of GnRH and Antide were observed under a light microscope and were presented in micrograph (A). Total tube length of the capillary-like networks after co-treatment of GnRH and Antide was quantified using North Eclipse software and was presented in bargraph (B). The relative value of total tube length in control group were normalized to 1 and presented as Ctrl. All data were derived from three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA).
Figure 7. GnRH regulates N-cadherin, cadherin-11, MMP2 and MMP9 expression in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated 100nM GnRH I and GnRH II. The cells were collected at 24h and 48h for mRNA isolation and protein extraction, respectively. The mRNA expression levels of N-cadherin, cadherin-11, MMP2 and MMP9 in these cells were tested using qRT-PCR and were presented in the bargraph (A). All data were generated by statistically analysis of three independent experiments and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraphs (n=3, P<0.05, Tukey test after one-way ANOVA). The cell lysates were loaded in SDS-PAGE and western blot was performed using N-cadherin, cadherin-11 (Clone 113H and 113E), MMP2 and MMP9 specific antibodies. All immunoblots were reprobed for α-tubulin to show the amount of lysates loaded in SDS-PAGE. The protein levels of N-cadherin, cadherin-11, pro-MMP2, pro- and active-MMP9 were presented in the chemiluminograms (B). All the experiments were repeated three times in independent occasions (n=3, P<0.05).
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N-cad mRNA/Gapdh (fold change)

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(n=3, P<0.05)
Figure 7. 4 N-cadherin and cadherin-11 regulate basal capillary-like network formation in HTR-8/SVneo cells.

HTR-8/SVneo cells were transfected with control siRNA or specific siRNAs against N-cadherin or cadherin-11. The cells incubated with transfection reagent only were used as vehicle control. After 48h, cells were trypsinized and placed on undiluted Matrigel basement for tube formation assay (see Chapter 2 section 2.11). Capillary-like network was observed under a light microscope. Representative capillary-like networks after siRNA transfection were presented in micrograph (A). Total tube length of the capillary-like cell networks after siRNA transfection was quantified using North Eclipse software and was presented in bargraph (B). The relative value of total tube length in vehicle control group were normalized to 1 and presented as Vehicle Ctrl. All data were derived from three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA). HTR-8/SVneo cells were pre-incubated with Ctrl IgG or N-cadherin perturbing antibody for 1h before tube-formation assay. Representative HTR-8/SVneo cell network after N-cadherin perturbing antibody incubation are presented in the micrograph (C) and the experiment was repeated three times (n=3).
Figure 7. MMP2 and MMP9 regulate basal capillary-like network formation in HTR-8/SVneo cells.

HTR-8/SVneo cells were transfected with control siRNA or specific siRNAs against RUNX2, MMP2 or MMP9. The cells incubated with transfection reagent only were used as vehicle control. After 48h, cells were trypsinized and placed on undiluted Matrigel basement for tube formation assay (see Chapter 2 section 2.11). Capillary-like network was observed under a light microscope. Representative capillary-like networks after siRNA transfection were presented in micrograph (A). Total tube length of the capillary-like cell networks after siRNA transfection was quantified using North Eclipse software and was presented in bargraph (B). The relative value of total tube length in vehicle control group were normalized to 1 and presented as Ctrl. All data were derived from three independent experiments (n=3) and are presented as the mean ± S.E.M. in the bargraphs. Significant differences were indicated by different letters in the bargraph (P<0.05, Tukey test after one-way ANOVA). HTR-8/SVneo cells were pre-incubated with MMP2/9 inhibitors for 1h before tube-formation assay. DMSO was used as the vehicle control of MMP2/9 inhibitors. Representative HTR-8/SVneo cell network after MMP inhibitors incubation are presented in the micrograph (C) and the experiment was repeated three times (n=3).
Figure 7. 6 N-cadherin, cadherin-11, MMP2 and MMP9 mediate GnRH-induced HTR-8/SVneo cell tube formation.

HTR-8/SVneo cells were transfected with control siRNA or specific siRNAs against N-cadherin, cadherin-11, MMP2 or MMP9. GnRH was added into culture medium 24h after transfection. The cells were tripsinized 24h after GnRH treatment and were seeded into the plate at a concentration of 2×10⁴ cells/well. The cells were incubated in 37°C for 24h. Capillary-like networks were observed under a light microscope and Images covering the entire tube network in each well were obtained. Total tube length of each network was quantified using Northern Eclipse software. The total tube length of GnRH-induced network after knockdown of N-cadherin and cadherin-11 were presented in the bargraph (A). The total tube length of GnRH-induced cells after knockdown of MMP2 and MMP9 were presented in the bargraph (B). The values of control siRNA group are normalized to 1 and presented as Ctrl siRNA in the bargraphs. All data in this figure were derived from four independent experiments and are presented as the mean ± S.E.M. in the bargraphs (n=4). Different letter indicates significant differences (P<0.05, Tukey test after one-way ANOVA).
CHAPTER 8: SUMMARY AND GENERAL DISCUSSION

8.1 Summary

As presented in my thesis, I examined the expression of GnRHR in first-trimester human placenta and trophoblastic cells. Based on my observation of abundant GnRHR expression in invasive EVT cells, I systemically investigated the role of GnRH in regulating trophoblast invasion and vascular remodeling through the reprogramming of mesenchymal cadherin expression and proteolytic dynamics.

As described in Chapter 3, I revealed the presence of GnRHR protein in the mononucleate cytotrophoblast layer, with elevated GnRHR expression in the cell column region during EVT differentiation. Moreover, abundant GnRHR protein was detected in the invasive EVT cell cultures by immunohistochemistry and Western blot analysis. These findings provided us with a rationale for studying the biological function of GnRHR and its roles in EVTs, invasion, and vascular remodeling.

As described in Chapter 4, I. Further experiments provided evidence that this up-regulation of N-cadherin was mediated by increased TWIST expression under the influence of GnRH signaling. Loss-of-function studies of N-cadherin and TWIST validated the biological roles of N-cadherin and TWIST in regulating trophoblast invasion. This part of my study demonstrated that GnRH regulates trophoblast invasion
via the modulation of TWIST-induced N-cadherin expression.

In Chapter 5, I further investigated the potential mechanism involved in trophoblastic proteolytic changes induced by GnRH. I demonstrated that GnRH induces RUNX2 expression in EVT cells. The endogenous inhibition of RUNX2 in trophoblastic cells resulted in decreased MMP-2 and MMP-9 expression and trophoblast invasion. From the data presented in this chapter, I demonstrated that the up-regulation of MMP-2 and MMP-9 expression by GnRH is likely to be mediated by RUNX2.


As presented in Chapter 7, my studies demonstrate for the first time that GnRH modulates the endothelial mimicry capability of EVT cells. This effect may be mediated by GnRH-induced reprogramming of the cell adhesion molecules N-cadherin and cadherin-11 as well as the gelatinases MMP-2 and MMP-9.

Taken together, the contents of my thesis have demonstrated that novel molecular mechanisms are involved in GnRH-regulated trophoblast invasion and vascular remodeling. The illustration of these mechanisms has provided insight into how the interactive molecular dynamics and manipulative cellular behaviors contribute to implantation and placentation during early pregnancy.
Figure 8. 1 A schematic diagram of the proposed mechanisms of GnRH regulating trophoblast invasion and vascular remodeling.
8.2 General discussion

Early implantation is a complicated but critical process that requires interactive coordination between multiple cell populations that appear during the first few weeks of pregnancy. The appearance of proliferative villous cytotrophoblast, fused syncytiotrophoblast, and invasive EVT cell populations not only provides the anchoring machinery between the embryo and uterus but also fulfills the requirement of material transmission within the placenta (Maltepe et al., 2010). Placenta development and trophoblast behaviors are tightly regulated by numerous growth factors, cytokines, and hormones (Knofler, 2010; Staun-Ram and Shalev, 2005), and GnRH is one of these important factors.

The presence of GnRH in the first-trimester human placenta and trophoblastic cells has been previously reported (Khodr and Siler-Khodr, 1978b; Seppala et al., 1980). The abundant expression of classical GnRH I in the human placenta has been reported to coincide with extensive trophoblast invasion in the first trimester (Prager et al., 1992; Siler-Khodr and Khodr, 1978). Here, we determined that GnRHR protein is immunolocalized in the villous cytotrophoblast and column EVTs. The peak level of GnRHR mRNA has been detected at 9-10 weeks gestation (Lin et al., 1995), and adequate GnRHR in first trimester EVTs may contribute to elevated EVT invasiveness. Moreover, both GnRH I and GnRH II proteins were immunolocalized to invasive EVTs in vivo and in vitro (Chou et al., 2004). The local existence of the GnRH-GnRHR system
in EVTs may provide evidence that an autocrine/paracrine role of GnRH may be involved in modulating trophoblast function. Interestingly, GnRH signals have also been detected at villous cytotrophoblast layer of first trimester human placenta (Chou et al., 2004). GnRH has been reported to inhibit cell growth and proliferation in pituitary cells and carcinoma cells (Kakar et al., 1997; Park et al., 2009). A previous study have demonstrated that Buserelin, a GnRH agonist, could inhibit villous cytotrophoblast cell proliferation and induce syncytiotrophoblast fusion in vitro, and this effect could be abolished by preincubation of GnRH antagonist Cetrorelix (Rama et al., 2004). This evidence indicates that local presence of GnRH-GnRHR system may exerts a distinguishable function to promote cell differentiation in villous trophoblast compare to that in EVT during early implantation.

Previous research has demonstrated that GnRHR mRNA could be detected in syncytiotrophoblast using in situ hybridization strategy (Lin et al., 1995). However, I was unable to observe positive GnRHR protein immunoreactivity in the first-trimester human syncytiotrophoblast layer in my immunohistochemistry study. It may due to reduced sensitivity of signal detection caused by the GnRHR antibody I used in immunohistochemistry procedure. Moreover, distinct membrane structure between apical membrane and basal membrane surface of syncytiotrophoblast may lead to uneven receptor localization, and a study has shown that Anx-2, a cholesterol associated protein, is only present at basement membrane but not at apical membrane in syncytiotrophoblast (Godoy and Riquelme, 2008). Similar phenomenon may apply to GnRHR, and adjacent
localization between cytotrophoblast and basal syncytiotrophoblast membrane may lead to undistinguishable GnRHR signals. Techniques such as electronic microscopy with advanced resolution may provide detailed evidences of the cellular localization of GnRHR in first-trimester human placenta.

Local expression of GnRHR in first-trimester human placenta may indicate regulatory roles of GnRH-GnRHR on early implantation and placentation. However, a study shows that no significant change of blastocyst development, implantation or maintenance of early pregnancy has been observed in a GnRHR null mice model (Wu et al., 2010). Another GnRHR mutation mouse model develops hypogonadotrophic hypogonadism but no report has demonstrated the potential role of gnrhr gene mutagenesis during early embryo development (Pask et al., 2005). It is possible that other growth factors, cytokines and hormones compensate the effect of GnRH after its signaling was abolished. For example, transactivation of EGFR is important to mediate many GnRH induced downstream molecule expression, including MMP2 and MMP9 (Liu et al., 2009; Roelle et al., 2003). Abundant EGF-EGFR expression in human trophoblastic cells (Bulmer et al., 1989) may provide alternative signaling during regulation and maintenance of early implantation.

Based on the evidence that both GnRH and its receptor are positively expressed in invasive EVTs, we hypothesized that GnRH potentiates EVT function during implantation and placentation, especially invasion and vascular remodeling. Similar to the previous report, I observed increased EVT invasiveness after GnRH treatment. The
differential expression of cadherins facilitates separate cellular motility. E-cadherin mediates strong cell-cell adhesion and low motility (van Roy and Berx, 2008); however, N-cadherin normally accompanies cellular invasive behavior in development and cancer metastasis (Derycke and Bracke, 2004). The loss of E-cadherin (Li et al., 2003) and gain of N-cadherin is a remarkable change during the differentiation process from the villous cytotrophoblast to EVTs. GnRH has been reported to suppress E-cadherin expression in ovarian cancer (Cheung et al., 2010), however, in E-cadherin positive choriocarcinoma cell lines, the regulatory role of GnRH to E-cadherin expression and cell invasiveness is still yet to be determined. Whereas, in E-cadherin-absent EVT cells, elevated N-cadherin expression was observed in my studies with the presence of GnRH, which promotes cell invasion. So far, no effective method has been established to maintain the column trophoblast in an “intermediate” differentiated status. Establishment of trophoblastic cell population that expresses both endogenous E-cadherin and N-cadherin may be benefit to study the transition process during villous cytotrophoblast differentiation towards invasive EVTs.

The transcription factor TWIST controls cell morphogenesis and mediates the switch from a low-motile cell type to a highly invasive phenotype (Yang et al., 2004). In my studies, I detected TWIST immunoreactivity in the EVT column and was the first to demonstrate that GnRH stimulates TWIST expression in trophoblasts. Evidence has shown that the regulatory role of TWIST in cell invasiveness requires the coordination of additional transcription factors such as SNAIL2 and SET8 (Casas et al., 2011; Yang et al.,
2012). However, loss-of-function of TWIST sufficiently attenuated N-cadherin expression and trophoblastic cell invasion, and a similar effect was also reported in cancer cells (Alexander et al., 2006; Rosivatz et al., 2002), showing that the TWIST involved transcriptional machinery may be distinct between different cell types and tissue types. Moreover, GnRH rapidly activates c-FOS/c-JUN phosphorylation in EVT cells according to my result. In renal carcinomas, phosphorylated c-JUN could be activated and form a dimerized AP-1 complex with c-FOS, leading to a transcriptional upregulation of TWIST gene in EMT process (An et al., 2013). In trophoblast, GnRH-induced TWIST expression may also have the participation of AP-1 transcription factor, and further experiment still need to be conducted.

GnRH I and GnRH II have been reported to regulate uPA/PAI-1 system balance in EVT cells (Chou et al., 2003a), suggesting that GnRH may regulate local proteolytic activity in the first-trimester trophoblasts. Moreover, besides inducing MMP2/9 expression, GnRH could also inhibit tissue inhibitors of metalloproteinases (TIMPs) expression in EVTs (Chou et al., 2003c), suggesting that GnRH play important role in proteinases activation. In ovarian cancer, β-catenin is involved in GnRH-induced MTI-MMP expression (Ling Poon et al., 2011). Beta-catenin and RUNX2 has been reported to be colocalized in the nucleus of osteoblast (Kode et al., 2014). Moreover, recruitment of β-catenin to RUNX2 promoter has been demonstrated using ChIP method (Gaur et al., 2005). Based on the evidences above, it is possible that β-catenin is involved in GnRH-induced RUNX2 expression. Additionally, cross talk between AP-1 and
RUNX2 has been reported in regulating bone remodeling related molecule such as MMP-13 (Selvamurugan et al., 2006). The involvement of AP-1 in basal and GnRH-induced RUNX2 expression in trophoblast still need to be further investigated.

Many cofactors have been reported to collectively regulate target gene expression with RUNX2. For instance, other bHLH transcription factors Hand1 and Hand2 have been reported to physically interact with Runx2 and regulate Runx2 mediated transcription in mouse osteoblast (Funato et al., 2009). Interestingly, Hand1 protein has also been reported to regulate mouse trophoblast precursor differentiation toward trophoblast giant cells (Scott et al., 2000). Moreover, transcriptional network between TWIST and RUNX2 in different cell types has also been studied. For example, TWIST has been shown to inhibit RUNX2 induced osteoblast differentiation by directly interacting with the RUNX2 DNA binding domain (Bialek et al., 2004). Another study has also reported inactivation of TWIST alters RUNX2-DNA binding affinity, whereas TWIST could promote RUNX2 expression in osteoblast (Yousfi et al., 2002). Whether the interactive network between these early developmental related transcriptional factors in other cell types are also present in trophoblast still need to be further investigated.

Many growth factors such as VEGF, platelet growth factor (PIGF) and angiopoietin (ANG1 and ANG2) are involved in angiogenic behaviors during early development and pathological conditions (Carmeliet and Jain, 2011). Other molecules such as VE-cadherin and integrin αvβ3 are also highly expressed in EVT cells. Previous reports have demonstrated that withdraw of GnRH agonist in IVF patients could decrease follicle
VEGF level to prevent ovarian hyperstimulation, suggesting that GnRH agonist might regulate angiogenic factor such as VEGF in the ovary (Ding et al., 2013). Based on the evidences above, the potential role of GnRH in the expression of VEGF and other angiogenesis related factors in placenta is worth to be demonstrated.

Several different study models have been widely used in placenta and trophoblast research (Carter, 2007; Genbacev and Miller, 2000). These study models include cell models such as primary trophoblastic cells, immortalized trophoblastic cell lines and trophoblastic stem cells, and animal models such as mice and non-human primates. In this study, I have used primary and immortalized EVT cells in vitro to study the biological role of GnRH in trophoblast behavior and gene profile change. The convenience of immortalized cell culture and gene-expression manipulation has made it accessible to directly target the molecules such as TWIST, RUNX2 and cFOS/c-JUN in studying basal and GnRH-induced trophoblast behaviors. Additionally, cells from primary EVT cells culture share large similarity with EVT cells in vivo and could reflect individual differences in responding of GnRH treatment in my study. However, behaviors of in vitro cell models could not fully mimic the physiological phenomenon in vivo. For example, two-dimension in vitro tube-formation assay could not fully mimic vascular formation in tissue with a three-dimension structure. Moreover, hormone concentration and half-life in physiological conditions in vivo may be different with experimental conditions in vitro, as the concentration of GnRH that induces target gene expression in immortalized cell lines (100nM) is much higher than its serum level (1-10nM). Studies
using animal models such as knockout mice could a good benefit in studying the role of specific molecules such as TWIST, N-cadherin and RUNX2 in early implantation and placentation.

Both GnRH I and GnRH II are expressed in extra-pituitary tissues and are known to exert regulatory effects in various reproductive tissues such as the ovary, mammary gland, testes, and placenta (Ramakrishnappa et al., 2005). Interestingly, only GnRH I and not GnRH II was detected in the syncytiotrophoblast layer in first-trimester human placenta (Chou et al., 2004), indicating that distinct trophoblast cell population may selectively express different components of the GnRH-GnRHR axis. GnRH II has been shown to exhibit a greater role in several biological functions, such as its anti-proliferative effect in endometrial and ovarian tissue, compared with GnRH I (Grundker et al., 2002). However, no significant difference in either the invasive capability or gene expression pattern was observed in my studies between the two forms of GnRH. The presence of a second form of the GnRH receptor (GnRHR II) in humans is still under debate because neither the full-length mRNA transcript nor the functional activated protein of GnRHR II has been isolated (Cheng and Leung, 2005). Chou et al reported that a GnRHR-specific antagonist, Cetrorelix, only abolished the biological function of GnRH I but not GnRH II in regulating MMP and the uPA system in EVT cells and decidual stromal cells. However, in my studies, a different GnRHR antagonist, Antide, completely inhibited the regulatory effect of both GnRH isoforms in the EVT cells. In a non-primate animal model, inhibition of the GnRHR signal by Antide is transient, whereas the suppression effect by
Cetrorelix is shown to be prolonged (Weinbauer and Nieschlag, 1993), suggesting that Cetrorelix may present a lower receptor disassociation rate. Prolonged receptor binding of Cetrorelix may lead to partial activation of GnRHR, as a study has reported that Cetrorelix exerts a similar effect to GnRH in inhibiting chorionic JAR cell growth in vitro (Horvath et al., 1995). Partial agonist effect of certain GnRH antagonist could be a potential limitation in my study, so knock-down of GnRHR by specific siRNA could further confirm the role of GnRHR in GnRH-induced down-stream gene expression and trophoblast invasion.

Different IVF protocols using both GnRH antagonist and GnRH agonist have been performed to optimizing the best outcome according to the variability between women (Huang and Rosenwaks, 2012). Recently, GnRH antagonist is replacing GnRH agonist to become the most widely used drug in ovarian stimulation to minimize ovarian hyperstimulation. Additionally, GnRH analog could alter endometrial receptivity by regulating endometrial gene profile (Chou et al., 2003b; Rackow et al., 2008). Whether usage of GnRH agonist during ovarian stimulation in IVF could regulate local trophoblast invasion and vascular formation is still yet to be determined.

As I systematically described in Chapter 1, section 1.2.6, many important growth factors, cytokines and hormones composed complicated signal networks in regulating trophoblast invasion and vascular remodeling through distinct mechanisms. In my current studies, GnRH has shown to be an important member in this signal network through its role of regulating mesenchymal cadherins and proteases expression. Additionally, GnRH
could potentially regulate other hormones secretion in placenta. For example, GnRH has been reported to stimulate prostaglandin secretion in placenta tissues and this effect is more potent on week 13-15 of gestation age (Siler-Khodr et al., 1986a). Accumulating evidences have shown that prostaglandin play important roles in establishing blastocyst-decidua interaction (Kennedy et al., 2007) and stimulating trophoblast invasion (Horita et al., 2007; Nicola et al., 2008), suggesting that GnRH may regulate early implantation and placentation via inducing prostaglandin expression. Moreover, GnRH has long been reported to stimulate hCG secretion in trophoblastic cells (Siler-Khodr et al., 1986b), and hCG could up-regulate GnRHR expression in these cells (Cheng and Leung, 2002). Additionally, hCG and its β-subunit has also been reported to stimulate trophoblast invasion(Lee et al., 2013) and low serum hCG may be a potential predictor of certain pregnancy related complication such as pre-eclampsia (Bahado-Singh et al., 2002). Administration of hCG during IVF procedure and elevated hCG production during early preganancy also contribute to pregnancy maintenance. The studies in my thesis have demonstrated that GnRH regulates trophoblastic cell invasion and vascular remodeling via the modulation of cell adhesion and proteolysis at the maternal-fetal interface. These results further revealed the underlying mechanism(s) by which GnRH contributes to early implantation and placentation. Furthermore, my studies also suggest that therapeutically targeting GnRH and its receptor may serve as a potential clinical approach in the treatment of infertility and implantation-related disorders such as preeclampsia.
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