# TWIST REGULATES E-CADHERIN AND N-CADHERIN EXPRESSION LEVELS IN DISTINCT HUMAN TROPHOBLASTIC CELL LINES *IN VITRO*

by

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# ABSTRACT

Cadherin gene family members are known to be involved in the differentiation of cytotrophoblasts of the human placenta. In particular, the regulation of cadherin expression is coupled with the development of an invasive phenotype and the formation of the multinucleated syncytiotrophoblast. To investigate further the mechanisms underlying the differential regulation of cadherins during these developmental processes, we have examined the role of the transcription factor known as Twist. Twist is a basic HLH (helix-loop-helix) factor which has been shown to regulate cadherin expression in a variety of human tissues under normal and pathological conditions. Using an siRNA strategy, I have determined that Twist regulates both E-cadherin and N-cadherin in distinct subtypes of human trophoblastic cells *in vitro*. In particular, suppression of Twist gene expression in poorly invasive BeWo choriocarcinoma cells by using Twist-specific siRNA resulted in a concomitant increase in E-cadherin mRNA and protein levels in these cells. In contrast, transfection of highly invasive extravillous cytotrophoblasts with Twist siRNA decreased N-cadherin mRNA levels in a concentration-dependent manner. Taken together, these observations indicate that Twist differentially regulates E-cadherin and N-cadherin in human trophoblastic cells, two cadherin subtypes that govern the differentiation of these cells along the non-invasive and invasive pathways respectively. Although, the results of my studies do not directly demonstrate this biological function of Twist, they support the speculation that alterations in Twist expression levels will result in cadherin-mediated disorders of pregnancy associated with aberrant trophoblast differentiation.

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# ABBREVIATIONS

AP-2 (Activator Protein-2)

bHLH (Basic Helix-loop-helix)

BCA (Bicinchoninic Acid)

CAMs (Cell Adhesion Molecules)

CAR (Cell Adhesion Recognition)

cDNA (Complementary Deoxyribonucleic Acid)

CSF-1 (Colony Stimulating Factor 1)

Cx40 (Connexin40)

DMEM (Dulbecco's Modified Eagle Medium)

DMSO (Dimethyl Sulfoxide)

dNTP (Deoxynucleoside Triphosphate)

DNA (Deoxyribonucleic Acid)

E2 (Estradiol)

ECM (Extracellular Matrix)

ECL (Enhanced Chemiluminescence)

EDTA (Ethylenediaminetetraaceticacid)

EGF (Epidermal Growth Factor)

EMT (Epithelial to Mesenchymal Transition)

ER (Endoplasmic Reticulum)

EVT (Extravillous Cytotrophoblast)

FBS (Fetal Bovine Serum)

GC (Giant Cells)

GM-CSF (Granulocyte/Macrophage Colony Stimulating Factors)

GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase)

hACTH (Human Chorionic Adrenocorticotropin)

hCG (Human Chorionic Gonadotropin)

hCT (Human Chorionic Thyrotropin)

HIF-1 (Hypoxia-inducible Factor-1)

IL-1beta (Interleukin-1Beta)

IP3 (Inositol 1, 4, 5-trisphosphate)

LIF (Leukemia Inhibitory Factor)

MAPK (Mitogen-activated Protein Kinases)

MBD3 (Methyl-CpG Binding Domain Protein 3)

MDCK cells (Madin-Darby Canine Kidney)

MMP (Matrix Metalloproteinases)

MTA3 (Metastasis Associated 1 Family Member 3)

$N_2$ (Nitrogen)

NC (Nitrocellulose)

NCBI (National Center for Biotechnology Information)

P4 (Progesterone)

PA (Plasminogen Activators)

PBS (Phosphate Buffered Saline)

PCR (Polymerase Chain Reaction)

PKC (Protein Kinase C)

PMSF (Phenylmethylsulphonylfluoride)

RISC (RNA-induced Silencing Complex)

RNAi (RNA Interference)

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

SDS (Sodium Dodecyl Sulphate)

siRNA (Short Interference RNA)

STBM (Syncytiotrophoblast Membrane)

TBS (Tris Buffered Saline)

TGF (Transforming Growth Factor)

TGF-β （Transforming Growth Factor-beta)

TNF-α (Tumor Necrosis Factor Alpha)

uPA (Urokinase-type Plasminogen Activator)

VCAM (Vascular Adhesion Molecule-1)

VE-cadherin (Vascular Endothelial-Cadherin)

VEGF (Vascular Endothelial Growth Factor)

VEGFR-1 (Vascular Endothelial Growth Factor Receptor-1)

# 1. Introduction

## 1.1 The Human Placenta

Placental development is dependent upon cytotrophoblast differentiation (Jurisicova et al., 2005). There are two important specialized structures critical for placental function: floating villi and anchoring villi, both of which are formed by the differentiation and proliferation of trophoblast cells during pregnancy (Aplin, 2006). After embryonic cytotrophoblasts invade into the maternal uterus, many trophoblastic villi are formed by the proliferation and differentiation of the cytotrophoblasts (Huppertz et al., 2005). The initial villi are termed primary villi (Cross et al., 2006). At this stage, the maternal spiral arteries are modified and connected to the vein of the endometrium in the space between the chorionic villus. In this way, the villi are surrounded by the maternal blood (Cross et al., 2006). As the primary villus continues to develop to the later stages, termed the secondary villus, a central core will be formed within the villus by extra-embryonic mesoderm cell differentiation (Cross et al., 2006). The fetal blood vessels then form within the core of the villus and are responsible for the fetal circulation (Cross et al., 2006). This allows for exchange of materials between the fetal blood which is inside the villus and the maternal blood, which is outside of the villus. These changes take place within the first three weeks of pregnancy (Aplin, 2006). The majority of the villi will be arrested at this developmental stage (Aplin, 2006). They are now termed floating villi (Aplin, 2006). However, some of the villi will continue to develop to form anchoring villi. In anchoring villi, the further differentiated cytotrophoblasts emanate from the distal side of the villus and invade deeply into the endometrium to anchor the placenta to the uterus and or can invade the maternal spiral arteries to remodel these blood vessels (Aplin, 2006). Some of these cytotrophoblasts will also spread out to contact cells coming out of other villi to eventually form the cytotrophoblast shell (Lala et al., 2002).

The placenta is an important tissue for pregnancy. It is responsible for hormone production, material exchange, nutrient synthesis and also acts as an immunological barrier thereby protecting the fetus against the maternal immune system (Bryan et al., 2006). The major hormones produced by the human placenta include human chorionic gonadotropin (hCG), human placental lactogen (hPL), human chorionic thyrotropin (hCT), human chorionic

adrenocorticotropin (hACTH), estrogens and progesterone, human placental growth hormone, insulin-like growth factors, endothelial growth factor, and relaxin (Bryan et al., 2006). In addition, many other protein products are produced by the placenta, but their functions are not completely understood (Bryan et al., 2006). Metabolic exchange is also important for successful pregnancy (Bryan et al., 2006). Oxygen, nutrients, water and other useful materials for fetal development are transferred from the mother to the fetus through the placenta. At the same time, metabolic waste including carbon dioxide will be transferred from the fetal to the maternal circulation. The placenta is also able to synthesize nutrients including glycogen, cholesterol and fatty acids which support fetal growth (Bryan et al., 2006). In addition, some maternal immunoglobulins may be provided to the fetus through the placenta, protecting the fetus against possible diseases or immune attack (Bryan et al., 2006).

As the placenta is an organ vital to pregnancy, and is critical for fetal development, any deficiency in its function has serious consequences for the health of both the mother and fetus (Bryan et al., 2006).

## 1.2 Preeclampsia

The word "*preeclampsia*" is derived from the Greek *eklampsis*, which means sudden flash or development (Hubel et al., 1997). Preeclampsia is also referred to as pregnancy toxemia or pregnancy-induced hypertension (Cnossen et al., 2006). It is a complication of the second half of pregnancy, labor, or early period after delivery (Cnossen et al., 2006). It is characterized by two forms: the maternal syndrome that includes hypertension and proteinuria, and the fetal syndrome that includes restricted fetal growth and poor development of the placenta (Cnossen et al., 2006). It is a common cause of both maternal and perinatal morbidities. Approximately 25% of all infants with a very low birth weight (<1500g) result from preeclamptic pregnancies (Yashwanth et al., 2006). The condition affects about 6-10% of all pregnant women (Cnossen et al., 2006). This rate could be higher in certain populations of women including those with multifetal gestation, or those with a previous history of preeclampsia (Sibai et al., 2006). At present, the only effective treatment for preeclampsia is delivery of the fetus (Sibai et al., 2006).

Although preeclampsia has been recognized for a long time, the molecular basis of this pregnancy disorder remains unclear. It is generally accepted that preeclampsia results from

poor placentation (Lyall et al., 2006). Failure of trophoblasts to appropriately invade into the uterus, fewer invasive trophoblasts, and/or failure to convert the spiral arteries and insufficient capacity of the uteroplacental circulation are often observed in the placental pathology of preeclampsia (Sankaralingam et al., 2006). A better understanding of the molecular and cellular mechanisms of trophoblast invasion would likely lead to the development of novel treatments for this debilitating disorder of pregnancy.

## 1.3 Trophoblast Differentiation

Trophoblast differentiation is dependent upon two mutually exclusive pathways: the syncytiotrophoblast (noninvasive pathway) and the extravillous cytotrophoblast (EVT) (invasive pathway) (Pollheimer et al., 2005). These differentiation processes continue until the end of the pregnancy (Pollheimer et al., 2005).

### 1.3.1 Noninvasive Pathway

Intercellular fusion of monocleate villous cytotrophoblasts cells leads to a multinucleated trophoblastic layer, the syncytiotrophoblast (Frendo et al., 2003). This layer is critical for pregnancy. The very first syncytiotrophoblast is able to penetrate or invade into the uterine epithelium to form the primary villus (Frendo et al., 2003). After this initial invasive step, the syncytiotrophoblast loses its invasive phenotype and forms the outer surface of the chorionic villus (Huppertz et al., 2006). It is a continuous cell layer which separates the maternal and fetal blood circulation (Huppertz et al., 2006).

The syncytiotrophoblast is a multinucleated cell which does not contain any lateral cell borders (Richart et al, 1961). Its nuclei are non-mitotic (Richart et al., 1961), although it is subject to constant renewal throughout pregnancy (Yesim et al., 2006). Fragments of the syncytio trophoblast layer are shed into the maternal circulation, and are termed syncytiotrophoblast membrane (STBM) (Gude et al., 2004). STBM are often abnormally increased in the circulation of women with preeclampsia (Cockell et al., 1997). However, the exact mechanisms underlying this cellular event under normal and pathological conditions are poorly understood. The syncytial trophoblast is maintained throughout pregnancy via the contribution of mononulceate mitotically active villous cytotrophoblasts which form a cell

layer directly underneath this terminally differentiated cell layers (Huppertz et al., 2004). This is achieved by the underlying cytotrophoblast cells continually fusing into the syncytiotrophoblast. During this process, plasma membranes are mainly involved (Castellucci et al., 1990). Without this compensatory contribution from the cytotrophoblasts, the syncytiotrophoblast would die within several days (Castellucci et al., 1990).

It has been demonstrated that when mononucleate villous cytotrophoblasts isolated from term placenta are cultured under normal culture conditions, these cells adhere to the culture plate within 3-12 hours, migrate to form aggregates within the first 24 h, and then in the next 24 hours of culture, form multinucleated syncytium (Kliman et al., 1986). The multinucleated syncytium formed in these primary cells can also synthesize and secrete a number of cell products as do their counterparts *in vivo*, including hCG, placental lactogen (Feinman et al. 1986), estrogen and progesterone (Kliman et al. 1986). Furthermore, differentiation along the syncytiotrophoblast pathway can be altered when the cytotrophoblasts are cultured under different culture conditions. For example, hCG treatment can promote the syncytialization of villous cytotrophoblasts derived from term pregnancy placenta (Zhou et al., 2001).

### 1.3.2 Invasive Pathway

The second trophoblast lineage involves the development of interstitial or endovascular cytotrophoblasts (Pollheimer et al., 2005). During the earliest stages of pregnancy, the cytotrophoblastic stem cells residing at the villous basement membrane lose their polarity, begin to proliferate and form cell columns in the anchoring villi (Caniggia et al., 1997). EVTs emanate from the outer most part of these cell columns, giving rise to the endovascular and interstitial invasive cytotrophoblasts (Pollheimer et al., 2005). Unlike tumor cells, EVTs stop proliferating as they undergo differentiation (Pollheimer et al., 2005).

The interstitial invasive cytotrophoblast migrates through and invades into the uterine tissues (Chandan et al., 2002). These cells penetrate the first third of the adjacent myometrium and anchor the placenta to the maternal endometrium (Chandan, et al., 2002). These cells will also differentiate into giant cells (GC) in deeper areas of the placental bed (Huppertz et al., 2006).

The endovascular invasive cytotrophoblast migrates towards the maternal uterine spiral arteries (Chandan et al., 2002). There, these trophoblasts replace the endothelial cells lining the

spiral arteries, acquire an endothelial cell phenotype and then degrade the muscle cell layer which is necessary to maintain blood vessel integrity (Chandan et al., 2002). This process is termed conversion (Aplin et al., 2006). Conversion lowers vascular resistance allowing the increased blood flow required to meet the demands of the fetus as pregnancy progresses (Aplin et al., 2006). In patients with preeclampsia, conversion of an insufficient number of maternal arteries is often observed (Aplin et al., 2006).

## 1.4 Defects of Trophoblast Differentiation in Preeclampsia

Defects of trophoblast differentiation are associated with preeclampsia, especially during the second and early third trimesters of pregnancy (McMaster et al., 2004). In preeclampsia, the anchoring villi are severely affected (Mcmaster et al., 2004). The interstitial invasion of trophoblast into the myometrium is shallow (McMaster et al., 2004). Endovascular invasion of trophoblasts into the maternal arteries is also insufficient compared to normal pregnancy. This is believed to be the result of cytotrophoblasts failing to differentiate and to gain a vascular phenotype (Zhou et al., 1997). It is therefore difficult to detect cytotrophoblasts in the maternal vessels of the placental bed of preeclamptic patients (Red-Horse et al., 2004). These observations have led to the proposal that poor cytotrophoblast differentiation is a major underlying cause of preeclampsia. Although preeclampsia is a complex syndrome, a better understanding of cytotrophoblast differentiation could lead to novel cell-based diagnostics and treatments for preeclampsia.

## 1.5 Regulation of Trophoblast Differentiation

The differentiation of cytotrophoblast along the two distinct and mutually exclusive pathways underlying the formation and organization of the human placenta is governed by a variety of hormonal, cellular and molecular factors.

### 1.5.1 Hormones and Growth Factors

#### 1.5.1.1 Roles of Growth Factors

Treatment of cytotrophoblasts isolated from first trimester placental tissues with activin A, leukemia inhibitory factor (LIF) and interleukin-1β (IL-1β) induces these cells to undergo differentiation along the invasive pathway (Morrish et al., 1998). Conversely, treatment with transforming growth factor (TGF) β1, TGFβ3, glucocorticoids and hypoxic culture conditions inhibit these cells from developing an invasive phenotype (Morrish et al., 1998). However, this is in contrast to villous cytotrophoblasts isolated from term placenta. Villous cytotrophoblasts undergo differention along the invasive pathway following treatment with epidermal growth factor (EGF), EGF-II, IGF-binding proteins-1 (IGFBP-1), and α1β1 integrin (laminin receptor) and hypoxia (Morrish et al., 1998). However, when primary culture of villus cytotrophoblasts are treated with granulocyte/macrophage colony stimulating factors (GM-CSF), colony-stimulating factor 1 (CSF-1), dexamethasone, and hCG, they undergo terminal differentiation and fusion to form syncytia (Morrish et al., 1998). In contrast, TGFβ1 treatment inhibits syncytial formation in these cell cultures (Morrish et al., 1998).

Furthermore, IGF is involved in regulating trophoblast turnover (Forbes et al., 2008). Recent studies have shown that IGF may regulate cytotrophoblast proliferation and syncytial formation through the MAPK pathway, whereas its effects on survival are mediated by the PI3K pathway (Forbes et al., 2008).

Taken together, these observations demonstrate specific roles for distinct growth factors in trophoblast differentiation and fusion.

#### 1.5.1.2 Roles of Hormones

Estrogen is one of the most critical hormones in pregnancy (Doria et al., 2006). During syncytiotrophoblast differentiation, estrogen levels increase (Yashwanth et al., 2006). Estrogen receptor is also expressed on the surface of cytotrophoblast cells (Yashwanth et al., 2006). Estradiol (E2) can promote morphological and biochemical differentiation of human

trophoblastic cells along the non-invasive differentiation pathway after 72-96 hours of treatment, which coincides with an increase in hCG levels in these cell cultures (Yashwanth et al., 2006).

Progesterone (P4) is a well-studied hormone that is critical for the recognition and maintenance of pregnancy (Maruo et al., 2003). This hormone is secreted by trophoblastic cells *in vivo* and *in vitro* (Maruo et al., 2003). P4 is capable of regulating trophoblastic cell differentiation and function in an autocrine and/or paracrine manner, particularly during the implantation (Maruo et al., 2003). In particular, P4 regulates maternal uterine function thereby regulating the ability of cytotrophoblasts to invade into and to interact with endometrial cells *in vivo* (Maruo et al., 2003). P4 is also capable of promoting the attachment and invasion of primary cultures of human extravillous trophoblasts (Dai et al., 2003), giant cells of mouse blastocyst cultured with the uterine endothelial cells, and inhibiting the formation of secondary trophoblast giant cells when the ectoplacental cone is in contact with uterine decidual cells of the mouse (Dai et al., 2003). P4 also inhibits the expression of IL-1 and IL-6, two cytokines responsible for promoting early implantation *in vivo* and the proliferation, differentiation and invasion of human villous cytotrophoblasts *in vitro* (Dai et al., 2001).

hCG is also produced by trophoblastic cells (Hoffmann et al., 2002). Similar to P4, this placental hormone also regulates trophoblast differentiation via alteration in the expression levels of IL-1 and IL-6 (Dai et al., 2001).

#### 1.5.1.3 Vasculogenic Factors

The family of vascular endothelial growth factors (VEGF) includes many family members, such as VEGF-B, VEGF-C, VEGF-D, and VEGF-F (Yamazaki et al., 2006). The receptors for these growth factors include VEGFR-1 (Vascular endothelial growth factor receptor-1), VEGFR-2, VEGFR-3 and neurophilin (Yamazaki et al., 2006). These growth factors and their receptors play key role in angiogenesis, vasculogenesis and endothelial cell growth via their capability to induce endothelial cell proliferation, promote cell migration, inhibit apoptosis, and induce permeabilization of blood vessels (Yamazaki et al., 2006). VEGF has also been assigned a key role in early placentation. As cytotrophoblasts differentiate and migrate into the myometrium, members of the VEGF family of growth factor are specifically expressed (Zhou et al., 2002). In particular, VEGFR1–3, soluble VEGFR-1, VEGF-A, VEGF-C

have been detected in EVTs. VEGF appears to also play a role in syncytial trophoblast formation, as first trimester cytotrophoblast treated with VEGF undergo syncytialization (Zhou et al., 2002). Furthermore, VEGF-A and VEGFR-1 expression levels are decreased in cytotrophoblasts isolated from the placenta of women with preeclampsia (Zhou et al., 2002).

### 1.5.2 Oxygen

A hypoxic environment is believed to play an important role in regulating trophoblast differentiation (Myatt et al., 2004). At 8 to 10 weeks of gestation, the oxygen tension in the placenta is around 18mm Hg in normal gestation, and at 12-13 weeks, the oxygen tension increases to approximately 61 mm Hg (Rodesch et al., 1992). It is now accepted that the placenta and fetus develop under hypoxic conditions during the early first trimester of pregnancy.

Oxygen has been found to promote a proliferative phenotype in trophoblasts *in vivo* and *in vitro* in either a direct or indirect manner. The culture of HTR-8/SVneo, an EVT cell line, under both serum-free and hypoxic conditions, increases proliferation and reduces the invasive capability of these cells (Kilburn et al., 2000). However, other studies have demonstrated that low oxygen tension promotes the invasive capacity of cytotrophoblastic cells, *in vitro.* Increased uPA (urokinase-type plasminogen activator) and uPA receptor in EVT cells, a proteolytic system involved in cell migration and invasion capability were detected in these cells cultured under hypoxic conditions (Graham et al., 1998).

The precise mechanism by which trophoblastic cells respond to low oxygen tensions, resulting in changes in their ability to undergo differentiation, are still not fully understood. HIF-1 (hypoxia-inducible factor-1) has been identified as a key regulator of a group of low-oxygen response genes in trophoblastic *in vitro* cells (Semenza et al., 2001).

### 1.5.3 Molecular Mechanisms

#### 1.5.3.1 Basic Helix-loop-helix Transcription Factor

The family of basic helix-loop-helix (bHLH) transcription factors plays important roles during the later stages of trophoblast differentiation (Meinhardt et al., 2005). The biological functions of bHLH are mediated by the formation of dimers which are able to recognize and

bind to specific DNA sequences within the promoter regions of target genes (Meinhardt et al., 2005).

Mash2 is a mammalian member of the achaete-scute family of bHLH transcription factors (Johnson et al., 1992). This transcription factor is strongly expressed in the extraembryonic trophoblast lineages and at distinct stages of embryonic development in mice (Rossant et al., 1998). Mash2 mutant mouse embryos die from placental failure (Johnson et al., 1992). Furthermore, spongiotrophoblast cells and their precursors are absent and the number of chorionic ectodermal cells is reduced in Mash2 mutant mouse placentas (Guillemot et al., 1994). These results indicate that Mash2 is a critical factor in the trophoblastic cell differentiation in mice.

Id1 and Id2, members of the Id family of HLH proteins which lack the basic DNA binding domain (Zebedee et al., 2001; Takeda et al., 2007), are also potential factors for regulating trophoblast differentiation. Members of the Id family regulate gene transcription indirectly by binding to other bHLH proteins, thereby inhibiting their transcriptional functions (Zebedee et al., 2001). This is a common mechanism used to regulate the activity of bHLH transcription factors (Zebedee et al., 2001).

Expression of Id-1 is high in undifferentiated trophoblast stem cells and is down-regulated during early differentiation. This suggests that Id-1 is involved in inhibiting the differentiation of trophoblast stem cells (Takeda et al., 2007). Id-2 mRNA and protein level are down-regulated as cytotrophoblasts differentiate along the non-invasive pathway (Janatpour et al., 2000). Conversely, inhibition of cytotrophoblast differentiation along this pathway results in the Id-2 expression being maintained (Janatpour et al., 2000).

#### 1.5.3.2 Other Transcription Factors

In a recent study, it was found that members of Activate Protein-2 (AP-2), including AP-2 alpha (TFAP2A), AP-2 beta (TFAP2B), AP-2 gamma (TFAP2C) were differentially expressed in trophoblasts at different stages of the mink gestation (Lopes et al., 2006). In particular, expression of AP-2 alpha was high and restricted in cotyledonary epithelial cells including binucleate trophoblast cells. Expression level of TFAP2B was low in binucleate trophoblast cells. TFAP2C was intermediatedly expressed mainly by mononucleate cells as compared with TFAP2A and TFAP2B. This may suggest that the AP-2 family is involved in

regulation of trophoblast differentiation and function in bovine placenta (Ushizawa et al., 2007).

#### 1.5.3.3 Heat Shock Protein B1 (HSPB1) and Mitogen-Activated Protein Kinase (MAPK) Intracellular Signalling Pathways

HSPB1 is differentially expressed in trophoblastic cells during differentiation in the mouse (Winger et al., 2007). Concomitantly, phosphorylation of HSPB1 and mitogen-activated protein kinase activated protein kinase 2 (MAPKAPK2) increases. Inhibition of MAPK 14 resulted in a reduction of HSPB1 phosphorylation and increases in trophoblastic cell death over differentiation. This result may suggest that HSPB1 and MAPK14 phosphorylation pathways are involved in trophoblastic cell differentiation (Winger et al., 2007).

#### 1.5.3.4 PPARgamma (Peroxisome Proliferator-Activated Receptor-Gamma)

In the human placenta, PPARgamma is differentiatedly expressed in the villous cytotrophoblast and syncytiotrophoblast as well as in the extravillous cytotrophoblastic cells (EVCT) along their invasive pathway and believed to be involved in trophoblast differentiation (Barak et al., 2008). The regulation function of PPAR on trophoblastic cells may be mediated by its ligand 15d-PGJ2, as 15d-PGJ2 inhibit trophoblastic cells invasion in a concentration-dependent manner in primary trophoblast cell cultures and trophoblast cell lines (Barak et al., 2008). This evidence suggests that PPAR gamma is a crucial regulator of trophoblast differentiation.

#### 1.5.3.5 Integrins

Integrins have been assigned key roles in trophoblast differentiation (Li et al., 2004). In trophoblastic columns, the expression levels of the integrin α1, α5 and β1 subunits are down-regulated from the proximal side to the distal end (Qin et al., 2003). These findings suggest that these integrins subunits are involved in the differentiation of EVT since cells at these two parts of the villus column have different invasive capacities (Wang et al., 2002). In support of this, function-perturbing antibodies directed against integrin α1 and β1 block the development of an invasive phenotype in human villous cytotrophoblasts *in vitro* (Damsky et al., 1993). In cytotrophoblasts derived from placenta of preeclamptic patients, the expression of the α6β4

and α1β1 integrin complexes are aberrantly regulated (Damsky et al., 1993). These cytotrophoblasts also fail to gain an invasive phenotype (Damsky et al., 1993). This provides further evidence that integrins may play a key role in the trophoblast differentiation. Many growth factors and cytokines released by the maternal immune system, decidual cells or by the trophoblasts are likely to play important roles in regulating integrin expression during trophoblast differentiation. For example, tumor necrosis factor alpha (TNF-α) up-regulates α1β1 and down-regulates α6β1 expression in differentiating human cytotrophoblasts *in vitro* (Damsky et al., 1993). TGF-β also stimulates the expression of several integrin subunits and suppresses the invasive phenotype of trophoblasts undergoing differentiation along the invasive pathway (Damsky et al., 1993).

### 1.5.4 Cell-Extracellular Matrix Interactions

Cell-extracellular Matrix (ECM) interactions are specialized connections organizing cells into tissues and coordinating their biological functions (Newby et al., 2006). The formation of ECM interactions by cells involves membrane-bound cell adhesion molecules (CAMs). The principal class of CAMs involved is integrins (Brevetti et al., 2006).

During cytotrophoblast differentiation and invasion, the ECM undergoes remodeling to form and preserve a functional placenta (Orecchia et al., 2003). ECM also plays a central role in trophoblast differentiation (Orecchia et al., 2003). For example, it has been demonstrated that villous cytotrophoblast interactions with decidual ECM induces the trophoblasts to undergo proliferation and differentiation (Aplin et al., 2006). Specific examples of ECM regulating trophoblast differentiation will be discussed below.

#### 1.5.4.1 Extracellular Matrix Degradation

ECM may also govern trophoblast differentiation via its degradation. Two main families of matrix-degrading proteases are activated: plasminogen activator (PA) and matrix metalloproteinases (MMPs) (Mignatti et al., 1996). However, PA activates some MMPs, which in turn degrade/activate a broad spectrum of ECM components (Mignatti et al., 1996). Urokinase-type plasminogen activator (uPA) is detected at the leading edge of invading mouse trophoblasts (Sharma et al., 1990).

The MMP family of extracellular proteinases regulates cell migration, invasion, proliferation, and apoptosis (Webster et al., 2006). MMP family members share common functional domains and activation mechanisms (Webster et al., 2006). MMPs are $Ca^2$+- and $Zn^2$+-dependent and are only active at a neutral pH (Webster et al., 2006). MMPs are inhibited by a family of tissue inhibitors of metalloproteinases (TIMPs) (Chirco et al., 2006). MMPs degrade most components of the ECM (Malemud et al, 2006). There are now >20 members of the MMP family (Chakraborti et al., 2003).

Cytotrophoblasts express high levels of MMP-9 (Alexander et al., 1996). In cultured cytotrophoblast cells, a neutralizing antibody directed against MMP-9 inhibits invasion and ECM degradation (Alexander et al., 1996). Production of MMP-9 by trophoblasts is down-regulated in the second half of gestation, corresponding to a reduction in their invasive capacity (Alexander et al., 1996). However, TIMPs are not detected in cultures of mouse blastocyst outgrowths (Zhou et al., 2001) nor in cultured human cytotrophoblasts, even those from late gestational placental tissues (Zhou et al., 2001). In contrast, TIMPs are found throughout gestation in both mouse and human decidua (Zhou et al., 2001). This implies that both MMPs and TIMPs play important roles in limiting the extent of trophoblast invasion. More direct evidence for an *in vivo* role for MMP-9 in trophoblast differentiation comes from studies using mice null-mutant for the Ets-2, a transcription factor that regulates MMP-9 expression, and MMP-9 genes (Yamamoto et al., 1998). Loss of Ets-2 leads to insufficient MMP-9 activity and death during early embryogenesis (Yamamoto et al., 1998). Failure of trophoblast migration and defects in placenta formation has been implicated in this embryonic lethal defect. In addition, TIMP-1 overexpressing transgenic mice (Alexander et al., 1996) and mice null-mutant for MMP-9 gene on some genetic backgrounds (Solberg et al., 2003) have confirmed that MMP-9 plays the same critical role in the regulation of trophoblast differentiation. Other MMPs are spatio-temporally expressed in the human placenta. For example, the expression of MMP26 in human placenta suggests a role for this MMP subtype in placentation (Qiu et al., 2005).

### 1.5.5 Cell-Cell Interactions

Inter-cellular junctions have been divided into six types: gap, adherens, tight, desmosome, hemidesmosome and focal contact (Fleming et al., 2000). Gap and adherens

junctions are two types of cell junctions assigned key roles in trophoblast differentiation (Houghton et al., 2005).

### 1.5.5.1 Gap Junctions

Gap junctions are specialized channels located on the surface of cells (Sosinsky et al., 2005). These junctions connect neighboring cells with each other and allow the exchange of small molecules including cyclic 3', 5'-adenosine monophosphate (cAMP), and inositol 1, 4, 5-trisphosphate (IP3) (Zhao et al., 2006). Connexins (Cx) are the hemi-proteins that are the structural components of gap junctions (Sosinsky et al., 2005). There are several connexin subtypes which exhibit distinct tissue distributions and have different functional properties (Dhein et al., 2002). Connexins have been detected in human cytotrophoblasts and syncytiotrophoblasts *in vivo* and *in vitro* (Cronier et al., 1997). The spatio-temporal expression of connexins in human trophoblasts has led to the proposal that gap junctions at least partly mediate the terminal differentiation of these cells. For example, Cx40 is expressed in the most proximal part of the EVT column where cells undergo proliferation. However as cytotrophoblasts undergo differentiation, leave the column, and invade into the maternal uterus, Cx40 is down-regulated (Cronier et al., 2002). Interestingly Cx40 is re-expressed in aggregates of trophoblast cells present in the decidua (Winterhager et al., 1999). Loss of Cx40 gene expression also inhibits trophoblast invasion (Nishimura et al., 2004). Cx43 expression has also been detected in villus cytotrophoblasts and in the syncytiotrophoblast (Frendo et al., 2003). Loss of Cx43 gene expression in cytotrophoblasts leads to poor aggregation and fusion (Frendo et al., 2003). Collectively, these observations indicate that gap junction formation promotes EVT proliferation and inhibits the differentiation of these cells along both the invasive and non-invasive pathways (Nishimura et al., 2004).

### 1.5.5.2 Adherens Junction

Adherens junctions are composed of a gene superfamily, cell adhesion moledules (CAMs), known as the cadherins (Zhou et al., 1996).

## 1.6 Cadherins

The cadherins are a gene family of integral membrane glycoproteins that mediate calcium-dependent cell-adhesion in a homophilic manner (Ozawa et al., 2006). E-cadherin was the initial member of this gene family to be identified (Hyafil et al., 1981). Subsequently, multiple cadherin subtypes have been identified in a wide variety of species spanning the evolutionary scale (Ozawa et al., 2006). The cadherin gene superfamily can be subdivided into four groups: 1) classical cadherins; 2) desmosomal cadherins that include desmocolins and desmogleins; 3) protocadherins that include u-Protocadherin and CNR-cadherin; 4) some other cadherin-related molecules, including 7TM-cadherin, T-cadherins and FAT-family cadherins (Angst et al., 2001). Classical cadherins include type I and type II cadherins. Type I cadherins include E (epithelial), N (neural), P (placental), and R (retinal) cadherin. Type II cadherins include human cadherin-5, -6, -8, -11, and -12 and also other type II cadherins found in mouse, rat, chicken, and *Xenopus* (Suzuki et al., 2006).

### 1.6.1 Protein Structure of the Classical Cadherins

The classical cadherins are composed of three structural and functional components: an extracellular domain, a transmembrane domain and a cytoplasmic domain (Derycke et al., 2004). The primary function of the extracellular domain is to bind calcium and to stabilize the structure of the classical cadherin (Derycke et al., 2004). The extracellular domain consists of several amino acid repeats, each of which is about 110 amino acids in length (Derycke et al., 2004), and these repeats are required to bind calcium ions. Varying features of these repeats include the number of residues and their composition. These characteristics are used to classify the distinct cadherins into subfamilies (Derycke et al., 2004). The classical cadherins are composed of extracellular domains containing five repeats. The classical cadherins have a specific amino acid sequence located in the first extracellular repeat that is referred to as the cell adhesion recognition (CAR) sequence, His Ala Val (HAV) (Derycke et al., 2004). The nonconserved amino acids located immediately next to the CAR site are responsible for the ability of these cadherins subtypes to interact with each other in a homophilic manner (Derycke et al., 2004)). However, many other cadherin subtypes, such as the desmosomal cadherins, are

capable of forming heterophilic interactions, at least *in vitro* (Chitaev and Troyanovsky, 1997; Marcozzi et al., 1998). The transmembrane domain is composed of about 24 amino acids, and connects the extracellular domain with the cytoplasmic domain, thereby anchoring the cadherins at the cell surface (Derycke et al., 2004). The cytoplasmic domains are the most highly conserved regions among the cadherins (Derycke et al., 2004). The cytoplasmic domains are linked to the microfilaments of the cytoskeleton via cytoplasmic proteins such as the catenins (Derycke et al., 2004). The cadherins bind directly to β-catenin and γ-catenin in a mutually exclusive manner. α-catenin then links both of these cadherin-catenin complexes to the cytoskeleton directly or indirectly through interactions with α-actinin (Derycke et al., 2004). Cadherin-catenin interactions modulate the adhesive capacity of cells (Derycke et al., 2004).

### 1.6.2 Human E-cadherin and N-cadherin Gene Structures

The human N-cadherin gene is located on chromosome 18q11.2 (Wallis et al., 1994). The human E-cadherin gene is located on chromosome 16q22.1. Both genes are composed of 16 exons and 15 introns (Wallis et al., 1994) (Fig. 1). Both genes also contain an E-Box sequence which is critical in transcription (Wallis et al., 1994). The N-cadherin gene contains an E-Box within intron 1 whereas the E-cadherin gene contains three E-Boxes within its promoter region (Yang et al., 2004) (Fig. 2). The presence of E-Box sequences in both cadherin genes suggests a common regulatory mechanism for their transcriptional activation.

### 1.6.3 Cell Biology of the Cadherins

#### 1.6.3.1 Cell Adhesion

The cadherins generally mediate cell-cell adhesion. Thus, the most basic function assigned to this family of CAMs is to maintain the integrity of multicellular, tissue structures (Suzuki et al., 2006).

### 1.6.3.2 Cell Signaling

N-cadherin is believed to be associated with cytoplasmic $Ca^{2+}$ (calcium ion) concentrations and/or activation of G-proteins and tyrosine kinases in either a direct or indirect manner (Doherty et al., 1991). Cadherin-11 is able to interact with the FGFR (fibroblast growth factor receptor) to trigger downstream signaling, which is involved in mouse growth cone progression (Boscher et al., 2008). Some intercellular signaling molecules including src family kinase proteins have been found at cell-cell junctions in epithelial cells, suggesting that E-cadherin and other cadherin members are involved in this kinase signaling pathway (Brady-Kalnay et al., 1995). Axon outgrowth can also be induced through interactions of N-cadherin with the EGF receptor and the subsequent activation of mitogen-activated protein kinases (MAPK) (Doherty et al., 2000). In addition, β-catenin is another candidate molecule participating in the regulation of cadherin function (Doherty et al., 2000). Collectively, these observations provide compelling evidence that cadherins are initiators of a variety of intracellular signaling pathways.

### 1.6.3.3 Cell Sorting

Homophilic cell-cell interactions mediated by cadherins are essential for cell sorting (Gilbert et al., 2006). Cell sorting means cells with a similar origin or function aggregate together to fulfill certain biological functions (Gilbert et al., 2006). This was first demonstrated in the early 1950s when cells from different amphibian blastophylla were found to be able to interact with other cells of a similar origin (Nose et al., 1988). It was then demonstrated that this cell recognition is based upon cadherin-mediated cell adhesion (Nose et al., 1988). Cell sorting is a process involved in organogenesis, during which tissues and organs are formed by the interaction of different cell subpopulations (Nose et al., 1988).

### 1.6.3.4 Epithelial to Mesenchymal Transition (EMT)

EMT is an important biological process (Lee et al., 2006), during which epithelial cells lose their polarity, become more motile and develop a mesenchymal phenotype (Lee et al., 2006). This is an important cell event during embryonic morphogenesis and tumorigenesis (Robson et al., 2006). Down-regulation of E-cadherin and up-regulation of N-cadherin expression are associated with EMT (Hatta et al., 1988; Yang et al., 2004). This switch in cadherin subtypes was suggested to be a marker of EMT progression (Agiostratidou et al., 2007).

### 1.6.3.5 Roles of the Cadherins in Tissue Morphogenesis

The process of tissue morphogenesis involves many of the cadherin-mediated biological functions described above. Thus, cadherins are also called morphoregulatory molecules (Redies et al., 1993). During embryogenesis, 20 different cadherins are expressed by neuroepithelial tissues and these cadherin subtypes are regulated in a spatial-temporal manner (Redies et al., 1993) suggesting key roles for the cadherins in neurogenesis. Before the formation of neuroectoderm, a switch from E-cadherin to N-cadherin expression occurs in cells destined to become neuroectoderm. This switch in these two cadherin subtypes is believed to be responsible for the formation of this cell layer (Duband et al., 1987). However, the contribution of cadherins in controlling morphogenesis is likely to be much more diverse and complex.

### 1.6.3.6 Role of Cadherin in Tumorigenesis

The cadherins are known to play central role in tumorigenesis (Tamura, 2006). Among the cadherin subtypes, E-cadherin and N-cadherin have been most extensively studied. Loss of E-cadherin-mediated cell-cell adhesion is frequently observed in tumor cell invasion and metastasis *in vivo* (Heimann et al., 2000). Association between reduced E-cadherin expression level and promotion of tumor invasion has been described in breast, squamous and pancreatic

endocrine carcinoma cells (Chetty et al., 2008). Loss of E-cadherin expression in noninvasive cells *in vitro* also promotes an invasive phenotype (Vleminckx et al., 1991). Conversely, up-regulation of E-cadherin in highly invasive prostatic and hepatocellular carcinoma cell lines decreases their invasive capacity and promotes cell-adhesion (Fransvea et al., 2008).

N-cadherin is also known to play an important role in tumorigenesis (Cavallaro et al., 2004). In a wide variety of carcinoma cells, N-cadherin is thought to stimulate migration and invasion (Bellusci et al., 1992), as increased expression levels of this CAM correlate with an invasive phenotype (Hazan et al., 2004). For example, when the breast cancer cell line MCF-7, which is E-cadherin positive is transfected with N-cadherin, an increase in motility and invasion is observed, although E-cadherin expression persists (Hazan et al., 2000). Conversely, in the retinoblastoma cell lines Y79 and Weri-Rb, a function-disrupting antibody specific for N-cadherin can inhibit the development of an invasive phenotype without affecting E-cadherin expression in these cells (Aken et al., 2002). This also demonstrates that gain of expression of N-cadherin, which is a mesenchymal marker, may promote motility and invasion of carcinoma cells independently of E-cadherin (Aken et al., 2002).

## 1.7 Cadherins and Pregnancy

### 1.7.1 Role of Cadherins in Embryogenesis

Mutiple cadherin subtypes have been shown to play central roles in the process of embryogenesis, owing to their diverse biological function (Lecuit et al., 2005).

Cadherin-11 expression is highly regulated during embryonic development (Borchers et al., 2001). In the mouse, during the early stages of embryonic development, cadherin-11 is spatio-temporally expressed between days 7.0 to 16.5 of pregnancy (Hoffmann et al., 1995). Cadherin-11 is thought to cooperate with N-cadherin in the formation and maintenance of epithelial somites (Horikawa et al., 1999). However, cadherin-11 gene knockout mice survive to the adult stage (Horikawa et al., 1999).

Vascular-endothelial cadherin (VE-cadherin) is expressed primarily in vascular endothelial cells (Dejana et al., 2001). The main function of VE-cadherin is to promote the

assembly and survival of vascular epithelial cells (Breier et al., 1996). During embryonic development, VE-cadherin regulates distinct aspects of placental and fetal development (Breier et al., 1996). VE-cadherin null-mutant mice die at mid-gestation resulting from vascularization defects in the anterior part of the embryo (Gory et al., 1999). In these mutants, the differentiation of endothelial cells was severely delayed in the early stages of vascular development (Gory et al., 1999).

E-cadherin is known to be a key regulator of embryonic development (Tepass et al., 2000). In particular, it has been demonstrated that a null mutation of the E-cadherin gene leads to mouse embryonic lethality (Larue et al., 1994). Furthermore, E-cadherin null mutant embryonic stem cells are not able to form trophectoderm epithelial or or a blastocyst cavity (Larue et al., 1994).

N-cadherin is essential for neural and somite morphogenesis and mesodermal germ layer morphogenesis (Warga et al., 2007). N-cadherin is first expressed by mesodermal cells of the gastrula of the mouse embryo (Derycke et al., 2004);. During stages of embryonic development, N-cadherin is restricted to the mesoderm but is present in neural tissue, lens and several other epithelial tissues at the later stages of embryogenesis (Derycke et al., 2004). During gastrulation, as cells undergo EMT, N-cadherin expression is up-regulated in a process concomitant with E-cadherin down-regulation (Hatta et al., 1986). Loss or gain of function studies demonstrated the importance of N-cadherin in embryonic development. For example, N-cadherin null-mutant embryonic stem cells are only able to form cartilage and neuroepithelium when transfected with N-cadherin (Larue et al., 1996). Expression of N-cadherin can partially rescue N-cadherin null embryos *in vitro* (Luo et al., 2001). However, N-cadherin knockout mice die at day 10 of gestation, exhibiting major cardiac defects and malformed neural tubes and somites (Radice et al., 1997). N-cadherin is also involved in several other aspects of embryonic development including osteogenic differentiation, bone development, myogenic differentiation and skeletal muscle formation (Zuppinger et al., 2000).

### 1.7.2 Role of Cadherins in Placentation and Trophoblast Differentiation

Cadherins family members play key roles in the process of trophoblast differentiation (Getsios et al., 1998).

Cadherin-11 is up-regulated during the syncytialization of cytotrophoblast cells *in vitro* (MacCalman et al., 1996). Ectopic expression of cadherin-11 in JEG3 cells, a mononuleate choriocarcinoma cell line, results in the induction of syncytial fusion of these cells *in vitro* (Getsios et al., 1998). Conversely, loss of cadherin-11 expression in BeWo cells will lead to the inhibition of the differentiation and fusion of these cells along the non-invasive pathway (Getsios et al., 1998).

Expression of VE-cadherin has been detected in the syncytiotrophoblast, fetal vascular cells, endovascular trophoblasts, EVTs, and the capillaries of terminal villi of human placentas (Leach et al, 1993). In the cytotrophoblasts of preeclamptic placenta, VE-cadherin is aberrantly up-regulated (Yan et al., 1997). In the labyrinth placenta of the mouse, VE-cadherin is also believed to play an important role in trophoblast differentiation (Zhou et al., 1997).

E-cadherin is also spatio-temporally expressed during the morphological and functional differentiation of human mononucleate villous cytotrophoblast cells forming multinucleated syncytial trophoblast (Coutifaris et al., 1991). E-cadherin expression is lost when cytotrophoblastic cells undergo differentiation or syncytialization. Conversely, E-cadherin protein levels have been shown to remain high in extravillous trophoblasts (Batistatou et al., 2007). In EVT cells obtained from preeclampsia patients, E-cadherin protein levels were found to be significantly reduced and the EMT was altered compared with normal pregnancy (Blechschmidt et al., 2007).

Other cadherin subtypes have also been assigned key roles in embryonic development and placentation. For example, P-cadherin, which was originally found to be expressed exclusively in the mouse placenta, is scarcely expressed in human placenta (Breier et al., 2000).

Collectively, these observations provide compelling evidence that cadherin family members play key roles in trophoblast differentiation and placentation. However, the mechanisms by which these CAMs are differentially regulated during trophoblast differentiation both along the invasive and non-invasive pathways remain to be elucidated.

## 1.8 Regulation of Cadherin Function and Expression

Cadherin function can be regulated via alterations in expression levels or through alterations in the stability of the cadherin-catenin complex (Nagafuchi et al., 2006).

### 1.8.1 Regulation of Stability of Cadherin-catenin Complex

#### 1.8.1.1 Catenin Phosphorylation

Catenins act as a bridge between the cadherins and the cytoskeleton (Semba et al., 2001). Alterations in catenin function or expression have distinct effects on cadherin-mediated adhesion (Nagafuchi 2006). β-catenin is a key molecule that has a central position in the formation/function of cadherin-cytoskeletal complexes (Lilien et al., 2002). The phosphorylation of β-catenin is an effective way by which cells promote cadherin-mediated adhesion (Sallee et al., 2006). Conversely, dephosphorylation of β-catenin promotes cadherin-mediated adhesion (Hu et al., 2001).

Another catenin subtype, p120, regulates cadherin-mediated adhesion in a similar manner (Xiao et al., 2006). p120 binds a certain regions of the cadherin molecule, which are required for clustering and dimerizing of these CAMs (Xiao et al, 2006). After the tyrosine or serine/threonine residues of p120 are phosphorylated, the cadherin-mediated adhesion formation is weakened (Xiao et al., 2006).

#### 1.8.1.2 Protein Kinase C (PKC)

PKC is an important intracellular signaling molecule (Ventura et al., 2001). It is responsible for phosphorylating a variety of proteins which control growth and cellular differentiation (Idris et al., 2001). PKC is able to regulate cadherin function in embryonic cells (Idris et al., 2001). For example, PKC treatment leads embryos to undergo a sustained increase in E-cadherin function (Idris et al, 2001).

### 1.8.2 Regulation of Cadherin Gene Expression

#### 1.8.2.1 Transcription Factors

Slug, Snail, ZEB-1and Smad (Sma and Mad related proteins) -interacting protein (SIP-1) belong to the Snail family of zinc-finger transcription factors (Cano et al., 2000; Comijn et al., 2001; Conacci-Sorrell et al., 2003; Grooteclaes and Frisch, 2000; Hajra et al., 2002). These

transcription factors bind specifically to an E-Box located in the E-cadherin gene promoter thereby playing an important role in regulating the expression of this gene (Karen et al., 2002). In particular, Snail, SLUG, ZEB-1 and SIP-1 have been found to suppress E-cadherin gene transcription (Karen et al., 2002). Thus, Snail has been found to be involved in the EMT process through its ability to regulate the expression of E-cadherin and other members of this family of CAMs (Blanco et al., 2002). Slug is also responsible for suppression of E-cadherin levels in carcinoma cells (Hajra et al., 2002) and the regulatory capability of this transcription factor on E-cadherin gene expression seems more potent than those observed with Snail (Hajra et al., 2002).

Other transcriptional factors which negatively regulate E-cadherin expression include Kaiso, E12/E47 and Twist (Cowin et al., 2005). The transcription factor Twist belongs to the bHLH family (Technau et al., 2003). Members of this family of transcription factors also specifically recognize E-Box motifs and regulate the expression of the downstream gene (Kewley et al., 2004). Twist has been shown to regulate both E-cadherin and N-cadherin expression in many kinds of carcinoma cells (Yang et al., 2004).

#### 1.8.2.2 Steroid Hormones

E2 reduces E-cadherin expression levels in breast cancer cells via a series of molecules including the ER (estrogen receptor) (Tanaka et al., 2006). ER was correlated with E-cadherin expression through its indirect regulation of metastasis associated family member 3 (MTA3). MTA3 is a novel component of the Mi-2/NuRD, which is a transcriptional repressor, a nucleosome-remodeling and a deacetylation complex (Tanaka et al., 2006). Using chromatin immunoprecipitation approaches, MTA3 was found to be able to directly regulate Snail transcription through methyl-CpG binding domain protein 3 (MBD3), which is present in the MTA3 complex and also the promotor element of the Snail gene (Tanaka et al., 2006). However, E2 could also up-regulate E-cadherin levels in human non-small cell lung cancer and human prostate cancer cells (Hershberger et al., 2005) and to reduce N-cadherin protein levels in rat pituitary GH3 cells (Heinrich et al., 1999). Thus, E2 could regulate cadherin expression via mutiple molecular mechanisms.

### 1.8.2.3 Transforming Growth Factor-β

TGF-β mediates EMT in mammary epithelial cells (Janda et al., 2002). Snail and SIP-1 are also activated, and in turn, E-cadherin expression is repressed `and N-cadherin expression is up-regulated at the transcriptional level (Janda et al., 2002). In recent studies, TGF-β has been shown to increase Slug and Snail expression and subsequently reduce E-cadherin expression in Langerhans cells (Herfs et al., 2008). These biological effects are mediated via its receptor and down stream effector molecules such as Smad proteins (Imamura et al., 2006). Smad proteins are downstream effector molecules of TGF- β receptor kinases and are considered to be responsible for mediating the biological action of TGF- β (Kang et al., 2000). Snail and SIP-1 are also regulated by TGF-β in rat hepatocytes, Madin-Darby canine kidney (MDCK) cells, and mice mammary epithelial cells (Comijn et al., 2001). These observations provide indirect evidence that TGF-β may also regulate cadherins through a series of intracellular signaling cascades.

Other molecules found to be involved in the regulation of cadherins include HER2/neu (ErbB2), EGFR, Ep-CAM and MUC-1/episialin (Marie, 2002). Activation of HER2/neu (ErbB2) represses E-cadherin transcription, leading to EMT in human mammary epithelial cells, while EGFR, Ep-CAM and MUC-1/episialin decrease E-cadherin function by disrupting its association with the cytoskeleton (Marie et al., 2002). Thus, the regulation of cadherins, both at the transcriptional level and protein function level, is a complex process.

## 1.9 Twist

The human Twist gene is located on chromosome 7p21 (Bourgeois et al., 1996). This gene is composed of two exons and three introns (Bourgeois et al., 1996). The first exon of the human Twist gene encodes a 202 amino acid protein, followed by a 536 bp intron and then the second exon (Bourgeois et al., 1996). The protein product of the Twist gene is detected as a 35kDa band by Western blot analysis (Bourgeois et al., 1996).

Twist was originally identified in *Drosophila* embryos. Embryos lacking Twist gene activity exhibited a twisted torso (Anderson et al., 1984). Twist has since been identified in humans (Wang et al., 1997), mice (Wolf et al., 1994), frogs (Hopwood et al., 1989), lancelets

(Yasui et al., 1998), nematodes (Harfe et al., 1998), leeches (Soto et al., 1997), zebra fish (Morin-Kensicki et a., 1997), jellyfish (Spring et al., 2000) and chickens (Tavares et al., 2001).

### 1.9.1 Twist Structure

Twist is a b-HLH transcription factor (Puisieux et al., 2006). The HLH domain contains two amphipathic helices which are used to mediate dimer formation and a nonconserved varying length loop which connects the two helices and is critical for maintaining the structure of the HLH domain (Howard et al., 1997). The basic amino acid-rich region preceding the first helix is responsible for DNA binding (Castanon et al., 2001). The bHLH domains are highly conserved within vertebrates (Yamada et al., 2005). Many mutations in the bHLH domain have been found to be responsible for embryonic defects in mammals (Howard et al., 1997). The Twist protein also contains sequences which allow it to be targeted to the nucleus (Howard et al., 1997). The nuclear targeting sequence is located within the bHLH domain, although alternative sites capable of performing this function have been identified (Ghysen et al., 1993).

bHLH proteins initially form dimers through the helix part, which then recognize and bind to the E-Box of target genes (5'-CANNTG-3') through the basic domain (Ephrussi et al., 1985). Members of the of bHLH transcription factor family form homodimers with each other or can form heterodimers with members of the E protein family (Kophengnavong et al., 2000). Different combinations of these transcription factors lead to more diverse functions and different DNA binding affinities (Kophengnavong et al., 2000).

### 1.9.2 Cell Biology of Twist

Twist is a critical transcription factor in development processes under normal and pathological conditions (Puisieux et al., 2006).

Twist-mutant *Drosophila* embryos fail to undergo gastrulation, do not form a mesoderm, exhibit a twisted torso and die before development is complete (Castanon et al., 1996). Similarly, in the mouse, Twist gene null-mutant embryos exhibit defects in the cranial mesenchyme, branchial arches, somites, and limb buds (Chen et al., 1995; Firulli et al., 2007)). In chicken, gain and loss of Twist function studies indicate that Twist1 can induce endocardial cushion cell proliferation and promote endocardial cushion cell migration during embryonic

heart valve development (Shelton et al., 2008). In humans, mutation in the Twist gene leads to Saethre-Chotzen syndrome. This syndrome presents as facial and limb abnormalities and mental retardation (Howard et al., 1997).

The Twist protein is expressed in a spatial and temporal manner during the early stages of embryonic development. In *Drosophila* embryos, Twist is initially expressed in the ventral-most cells of the embryo during the blastula stage (Leptin et al., 1991). After gastrulation, Twist is differentiatlly expressed in distinct mesodermal segments (Bate et al., 1993). In later development, the mesodermal cells which are guided to different cell differentiation lineages depend on their different Twist expression levels (Castanon et al., 1996). Thus in early embryonic mesodermal development, Twist expression level is thought to govern the developmental fate of cells. In the adult, significant levels of Twist are only found in some progenitor muscle cells (Bate et al., 1991).

Mice null-mutant for the Twist gene exhibit premature osteoblast differentiation suggesting a key role for Twist in this cellular event (Bialek et al., 2004). Twist overexpression inhibits osteoblast differentiation. This regulation is mediated via the interaction of Twist with another transcription factor, Runx2, which is required for osteoblast differentiation (Bialek et al., 2004). Twist is believed to also participate in neurogenesis. For example, mouse embryos null-mutant for the Twist gene exhibit severe defects in closure of the cephalic neural tube (Chen et al., 1995).

Up-regulated mRNA and protein levels of Twist are associated with malignant transformation and tumor development, in a wide variety of carcinomas, inluding gastric, prostatic, bladder, cervical, ovary, pancreatic, breast and hepatocellular cancers. It has been suggested that Twist may have a diagnostic use in cancers (Yuen et al., 2007). Twist is generally thought to promote carcinoma development through induction of EMT (Yang et al., 2006). This function might be mediated via the regulation of several other factors independently involved in EMT, such as E-cadherin and N-cadherin (Yang et al., 2006).

Twist is also involved in apoptosis (Maestro et al, 1999). Twist is able to inhibit oncogene (ARF) and p53-dependent cell death (Maestro et al., 1999). These observations have led to the proposal that Twist could act as an anti-apoptotic factor, another hallmark of cancer.

### 1.9.3 Twist Regulation of E-cadherin Gene Expression

Twist is a repressor of E-cadherin gene expression (Rosivatz et al., 2002). During EMT, E-cadherin mediated adhesion between epithelial cells is decreased, resulting in the loss of an epithelial phenotype and the concomitant development of a mesenchymal phenotype (Kang et al., 2004). A hallmark of EMT is a switch in expression from E-cadherin to N-cadherin (Hey et al., 1995). Twist has since been shown to decrease E-cadherin expression in carcinomas of the prostate (Yuen et al., 2007) and breast (Vesuna et al., 2008). A similar observation has also been made in the EMT switch during *Drosophila* gastrulation (Yang et al., 2004). However, Twist represses E-cadherin expression directly through an E-Box present in this gene during this development process (Yang et al., 2004).

### 1.9.4 Twist Regulation of N-cadherin Gene Expression

Twist has also been found to be capable of regulating N-cadherin gene expression in prostatic and gastric cancers (Yang et al., 2007). Using Twist and Snail null mutant *Drosophila* embryos, Twist has been found to be essential for the initiation of N-cadherin expression during gastrulation, whereas Snail was shown to be required for the increased expression of this cadherin subtype after gastrulation (Yang et al., 2004). Aberrant N-cadherin mRNA levels have been found in diffuse-type gastric carcinomas, suggesting that Twist regulates N-cadherin during the development and progression of this cancer to later stages of the disease (Rosivatz et al., 2002). Up-regulation of Twist expression in gastric cancer cells resulted in increased expression of N-cadherin expression and decreased E-cadherin expression. Conversely, suppression of Twist levels induced down-regulation of E-cadherion expression (Yang et al., 2007). Similar to E-cadherin gene expression, the trancriptional activity of N-cadherin gene is regulated by Twist via interactions with E-Box motifs in this gene. For example, inhibition of Twist expression has been shown to regulate N-cadherin mRNA expression levels in PC-3 prostate carcinoma cells via its ability to interact with the E-Box element located within the first intron of the N-cadherin gene (Alexander et al., 2006).

## 1.10 Trophoblastic Cells

### 1.10.1 BeWo Choriocarcinoma Cell Line

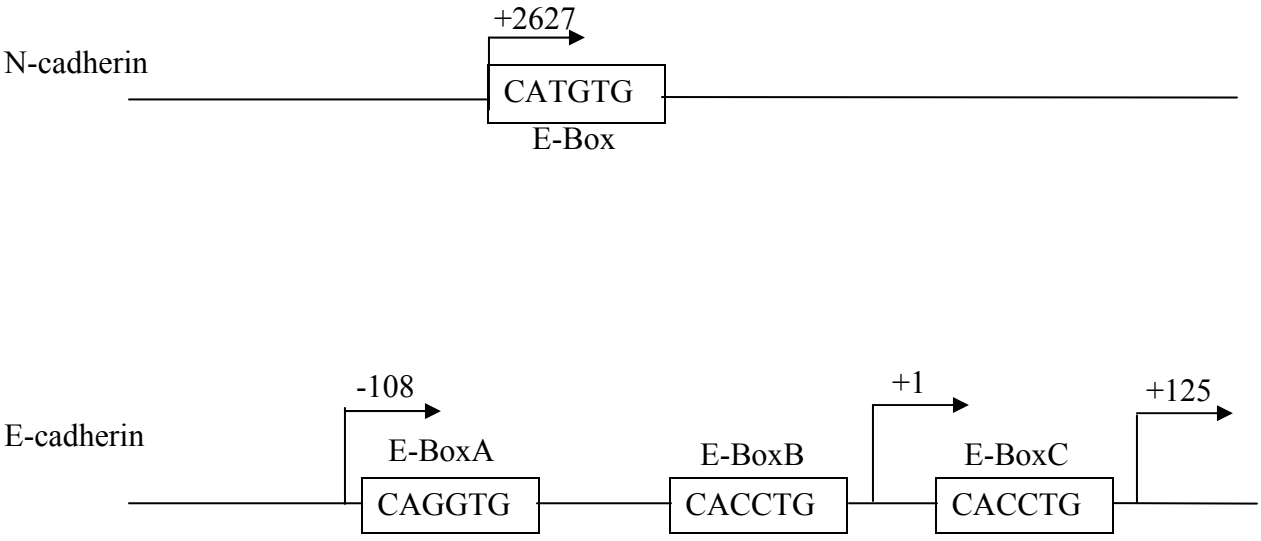
BeWo choriocarcinoma cells are a cell line derived from a human gestational choriocarcinoma (Lanoix et al., 2006). This cell line possesses many biological features similar to mononucleate cytotrophoblasts, including the secretion of various placental hormones such as hCG (Al-Nasiry et al., 2006). BeWo cells can be induced to undergo syncytialization in response to cAMP (Wice et al., 1990). They have been widely used as *in vitro* models for trophoblast intercellular fusion and differentiation (Burres et al., 1986; Hohn et al., 1992; Cohran et al., 1996; Kudo et al., 2004) and in toxicology studies (Burres, 1987). An advantage of using BeWo cells to study the biological functions of cytotrophoblasts *in vitro* is that they are comparably more stable with respect to inter-patient variability and other confounding variables associated with primary cultures of villous cytotrophoblasts (Lanoix et al., 2006).

### 1.10.2 EVT Cultures

The immortalized EVT cell line, HTR-8/SVneo trophoblast cell line, is derived from human first trimester placenta explant cultures and immortalized using SV40 large T antigen (Graham et al., 1993). This immortalized EVT cell line also shares many of the phenotypic and functional characteristics of the parental primary cell cultures. For example, they are morphologically similar (Graham et al., 1993). They are both highly invasive to uterine wall. Both of them are positive for cytokeratin, which demonstrates their epithelial identities. Thus, this immortalized EVT cell line provides a valuable *in vitro* model for studying EVT cell biology. In particular, this cell line has been used to study the mechanisms underlying the proliferation, migration, and invasion of EVT (Chakraborty et al., 2002).

Figure 1. E-Box regulatory elements within the human N-cadherin and the E-cadherin gene.

ATG is designated as +1.




Source: E-cadherin intron 2 contains cis-regulatory elements essential for gene expression. *Development*-June. *2005.*

# 2. Hypothesis

The switch from E-cadherin to N-cadherin expression during the development of an invasive phenotype in human trophoblastion cells is mediated by the bHLH transcription factor, Twist.

# 3. Proposed Research

Aim 1:

To determine whether loss of Twist expression increases E-cadherin mRNA and protein levels in poorly invasive BeWo carcinoma cells *in vitro*.

Aim 2**:**

To determine whether loss of Twist expression decreases N-cadherin mRNA and protein levels in highly invasive EVTs *in vitro*.

# 4. Materials and Methods

## 4.1 Maintenance of Cell Lines

### Passaging of Cells

To passage both cell lines, BeWo cells and EVTs, the culture medium was first discarded. Sterile PBS was then used to wash the cells twice, after which 3ml of Trypsin/EDTA (Ethylene diamine tetraacetic acid) solution (GIBCO) were added to the culture flasks or plates (Becton Dickinson and Co, Franklin Lakes, NJ, USA). The plates were incubated with trypsin for 5 minutes or until the cells had fully detached. An equal volume of culture medium was added to the plates. The cell suspension was transferred to a sterile tube and centrifuged at 800 x g for 5 minutes. After centrifugation, the supernatant was discarded and the cell pellet resuspended in 8 ml culture medium and transferred to new sterile 100 $mm^2$ plates. The cells were then cultured under standard culture condition until further passaging.

To preserve stocks of these cell lines, aliquots of BeWo cells and EVTs were frozen in liquid nitrogen ($N_2$). To do so, the cell pellets were resuspended in 1.5-2 ml culture medium containing 10% dimethyl sulfoxide (DMSO) and placed in liquid $N_2$. To reactivate these frozen cell aliquots, the cells were thawed as follows: the cells were removed from the freezer and pre-warmed at room temperature for 1 minute. They were then transferred to a 37ºC water bath for 1-2 minutes until fully thawed. Culture medium (about 1-2ml) containing the cells were slowly transferred from the storage tubes into a culture flask (25$cm^2$). Approximately 3-4ml of freshly prepared culture medium containing 10% FBS was then added to each flask to dilute the freezing medium. The cells were then cultured under standard cell culture conditions as described above.

### Culture Conditions

Immortalized EVTs were cultured in Dulbecco's Modified Eagle's edium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc. Burlington, ON, Canada) and 1% penicillin and streptomycin (100 IU/ml and 100µg/ml, respectively). BeWo cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured in Ham's Nutrient Mixture (F-12) supplemented

with 10% FBS and 1% antibiotics (as described above). Both cell lines were cultured under normal conditions (humidified incubator with 5% carbon dioxide at 37ºC) in 60 $mm^2$ plates.

## 4.2 Development of an siRNA Strategy to Target Twist

Background Theory: An siRNA strategy was employed to inhibit Twist expression in human trophoblastic cells. RNA interference (RNAi) is dependent upon the introduction of double stranded, synethic short RNA into cells, which are able to silence homologous gene expression (Scaringe et al., 2004). Initially, this techenique was developed in lower species including *Drosophila*, nematodes, fungi and plants but has since been shown to be effective in mammalian cells (Grabarek et al., 2003). It may even have a novel function in anti-viral defense of mammalian cells by modulating transposon activity (Grabarek et al., 2003).

siRNA binds to the RNA-induced silencing complex (RISC), which is a form of ribonuclease (Grabarek et al., 2003). When the siRNA complex is guided to the endogenous homologous mRNA, the RISC component is able to cleave in half the endogenous mRNA transcript (Yan et al., 2005). In this way, endogenous gene expression is inhibited at the mRNA level.

Design of siRNA specific for Twist: Strict criteria for the development of effective siRNA have been established and were used for the development of Twist siRNA in my studies. The approaches employed are summarized in the flow diagram presented in Fig. 2. The first step in the design of siRNA oligonucleotides is to examine and exclude the sequence between the 50-100 bases downstream of the start codon. This is owing to the sequence close to the start codon being rich in motifs that are easily recognized by binding proteins. Thus, the further the sequence is from the start codon, the less likely those other regulatory proteins will compete for the selected binding site.

Secondly, a search for a 23-nucleotide sequence that includes AA (N19) TT (N can be A, T, C, G) is performed. Other nucleate sequences that could be acceptable in decending order are: AA (N21) or CA (N21). If these sequences do not exist in the target gene, a dTdT overhang can be synthesized on to the 3' end of the antisense sequence in order to obtain the best transfection efficiency. This way, a nucleotide sequence of 19 bases with a dTdT overhang

at each side could be generated. However, if the 5' antisense sequence recognizes the target gene, the region close to the 3' of the antisense chain does not necessarily have specificity for the target gene (Kurreck 2006). The percentage of GC content in the siRNA sequence should be limited to 30-70%, as GC affinity is different from that of the AT bases (Kurreck 2006).

Finally, the sequence of siRNA must be specific for the target gene and not be able to recognize any other gene, particularly any other members of a gene family. Transfection efficiency and biological effects would be greatly affected if multiple genes were targeted (Kurreck 2006).

Following these design criteria, the siRNA oligonucleotide sequences selected for human Twist and used in my studies are as follow: GAA CAC CUU UAG AAA UAA AdTdT; Antisense sequence as UUU AUU UCU AAA GGU GUU CdTdT; The target sequence in native Twist RNA transcript was AAG AAC ACC TTT AGA AAT AAA; Control nonsense siRNA had a sense sequence of UUC UCC GAA CGU GUC ACG UdT dT, and antisense sequence of ACG UGA CAC GUU CGG AGA AdT dT. These siRNA constructs were prepared by Qiagen (Valencia, CA). To prepare a stock solution of siRNA, the lyophylised siRNA sample was resuspended in siRNA Suspension Buffer (RNase free water) to obtain a final stock solution with a concentration of 20μM. The siRNA stock solution was incubated at 90ºC for 1 minute followed by 37ºC for 60 minutes before being aliquoted and stored at -20ºC for subsequent use.

Transfection of Trophoblastic Cells with siRNA Owing to the different growth rates, BeWo cells were plated at a cell density of $4x10^5$ cells whereas EVTs were initially seeded at a density of $1x10^5$ cells per 60 $mm^2$ plates. These cells were then cultured for a further 24 hours before transfection with siRNA. After the cells had reached 50%-80% confluency, an aliquot (14μl) of the siRNA stock solution containing 2μg siRNA was diluted with 8μl Enhancer R and 78μl EC-R buffer. This reaction mixture was vortexed for 10 seconds and incubated at room temperature for 5 minutes, after which 10μl transmessenger Transfection Reagent was added to each tube. The mixture was vortexed for 10 seconds followed by incubation at room temperature for a further 10 minutes in order to allow the siRNA transfection complex to form. During this incubation period, a 2x volume of sterile PBS ($Ca^{2+}$ and $Mg^{2+}$ free) was used to wash the cells twice. Then, an aliquot of DMEM (900μl) without FBS or antibiotics was added to the transfection reaction mixtures. The reaction mixture was mixed before finally being

placed into the culture dishes. The cells were incubated with this siRNA transfection reaction mixture under standard culture conditions for 3 hours. The reaction complex mixture was removed and the cells washed with PBS before being replaced with 3ml of fresh culture medium containing 10% FBS and antibiotics. Cells were harvested after 12, 24 or 48 hours of culture under these experimental conditions.

## 4.3 Analysis of E-cadherin, N-cadherin, Twist and GAPDH mRNA Levels in Human Trophoblastic Cells

### 4.3.1 Total RNA Extraction

Total RNA was extracted from the trophoblastic cell cultures using TRI Reagent (Bio/Can, Mississauga, ON, Canada) according to a protocol provided by the manufacturer.

An aliquot (1ml) of TRI Reagent was added to each plate. The reaction was allowed to proceed for 5 minutes at room temperature. The reaction mixture was then transferred to a clean 1.5ml Eppendorf tube. Chloroform (Bio/Can Mississauga, Canada) (200µl) was added to each of the cell lysates, and vigorously shaken to allow the two solutions to mix with each other. The resultant homogenate was allowed to stand at room temperature for 2-15 minutes before being centrifuged at 12,000 x g for 15 minutes at 4ºC. The aqueous phase was then transferred to a fresh microcentrifuge tube after which isopropanol (Bio/Can, Missisauga, Canada) (500µl) was added in order to precipitate the total RNA present in each sample. After standing at room temperature for 10 minutes, the total RNA present in each sample was precipitated by centrifugation at 12,000 x g for 8 minutes at 4ºC. The supernatant was removed and the RNA pellet was washed with 75% ethanol (1ml), followed by vortexing and centrifugation at 7500 x g for 5 minutes at 4ºC. The RNA pellet was then briefly air-dried until all of the residual ethanol had evaporated. The RNA pellet was then dissolved in RNAase-free water and incubated at 65ºC for 10 minutes. These RNA solutions were stored at –80ºC for subsequent use in my RT-PCR analysis.

### 4.3.2 First Strand cDNA (complementary deoxyribonucleic acid) Synthesis

A First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Oakville, ON, Canada) was used to synthesize template cDNA from the RNA extracts prepared from the

trophoblastic cell cultures. The concentration of the RNA in each stock solution was analyzed by using a UV spectrophotometer at a wavelength of 260 nm. RNAase-free water was used to dilute an aliquot (1µg) of total RNA sample to obtain a final volume of 8µl with a concentration of RNA of 0.125µg/µl. The resultant RNA solution was incubated at 65ºC for 10 minutes before being chilled immediately on ice. An aliquot containing 5µl first-strand reaction mix, 1 µl Not I-d (T) 18 primers, 1 µl DTT (dithiothreitol) solution was added to the 8µl RNA solution. First strand cDNA synthesis was performed for 1 hour at 37ºC, after which the mixture solution is stored at –20ºC for subsequent use for my RT-PCR analysis.

### 4.3.3 RT-PCR

For each PCR reaction, $H_2O$ (13.87µl), Q-solution (Betaine 5M) (5µl), 10x PCR buffer (2.5µl), dNTP (deoxy-nucleoside 5'-triphosphate) (0.5µl), Forward Primers (1µl), Reverse Primers (1µl) specific for E-cadherin, N-cadherin, Twist or glyceraldhyde-3-phosphate dehydrogenase (GAPDH), Hot Star Taq 0.13µl and 1µl first strand cDNA were mixed together in a PCR tube. The Q-solution, dNTP mix, 10xbuffer and Hot Star Taq were all purchased from Qiagen Inc. After being allowed to stand for 1-2 minutes to enhance the mixture of these solutions, the PCR tubes were placed in a thermocycler.

When the effective and specific primers for those genes were designed, a 19-20 base pair sequence containing 50-60% (G+C) were selected. Then the primer sequence identified in each gene was BLASTED against the genomic database available at NCBI (National Center for Biotechnology Information). The nucleotide sequences for the primers for Twist, E-cadherin, N-cadherin, GAPDH, identified in the human mRNA sequences deposited in GenBank (National Centre for Biotechnology Information, Bethesda, MD) are shown in Table 1.

When an optimal PCR annealing temperature was determined, a series of increasing annealing temperatures were employed in the PCR reaction for each specific gene. The resultant the PCR products were resolved by electrophoresis in an agarose gel and stained with ethidium bromide (0.5mg/L). The brightness of the corresponding bands was compared and the temperature giving out the brightest bands are selected for the following studies (Table 1.).

To optimize the number of PCR cycles, PCR reactions were performed at increasing cycle numbers and PCR products were resolved by electrophoresis. The products were visualized by ethidium bromide staining and quantitated with computer software. A PCR curve was drawn with cycle numbers plotted against the log of the signal (Fig. 3). This curve is

composed of two parts: the exponential (linear) range and the plateau phase. During the exponential range, there is excess of reagents and active enzyme, so the PCR products are accumulating at a constant rate. During the plateau phase, the reaction component are not in excess, so there is little or no net increase in the levels of PCR product. For a meaningful RT-PCR result, the number of cycles should be within the linear range, during which time, PCR products are accumulating at a constant rate. As the PCR reaction product is very sensitive to slight variations in initial amplicafication conditions during the period before the exponential phase, the cycle number in this zone should be avoided. Finally, the individial PCR reaction with the chosen cycle number should be able to provide a visible band. Using this method, the optimized PCR cycle numbers for the primers of GAPDH, E-cadherin, N-cadherin and Twist were identified as 20, 30, 24, 30, respectively (Table 1).

After each PCR reaction, an aliquot (10μl) of PCR products was electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. The intensity of ethidium bromide staining was analyzed by using a UV densitometry imaging system (Biometra, Whiteman Co., Gottingen, Germany). A volume count of the each PCR product was determined using Scion Image computer software (Scion Image Co., Frederick, MD). For each PCR product, GAPDH was used as an internal control. The ratio of intensity of ethidium bromide staining of the target genes was normalized to that of the corresponding GAPDH PCR products

## 4.4 Analysis of E-cadherin, N-cadherin, Twist and β-actin Protein Expression Levels in Human Trophoblastic Cells

### 4.4.1 Total Protein Extraction

Cell lysis buffer was prepared by supplementing Cell Extraction Buffer (50mM Tris-HCl, pH 7.0, 1.5mM $MgCl_2$, 10mM KCl, 0.2 mM EDTA, 5% glycerol) with 1mM phenylmethylsulphonyl fluoride (PMSF) and 1x protease inhibitor cocktail (chymotrypsin 1.5μg/ml, thermolysin 0.8μg/ml, papaln 1mg/ml, pancreatic extract 1.5μg/ml and trypsin 0.002μg/ml) immediately prior to use. Cold PBS was used to wash the cells twice. Cells cultured in 60 $mm^2$ plates were scraped with a rubber spatula before an aliquot of lysis buffer (10mM Tris-HCl, pH7.5, containing 0.5% Nonidet P-40, 0.5 mM $CaCl_2$, and 1.0mM PMSF)

(100µl) was added to each plate and the reaction allowed to proceed for 30 minutes. During this incubation period, the plates containing the cells and lysis buffer were placed on a laboratory shaker at 4ºC. After the cells were completely lysed, the total protein extracts were transferred into micro centrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4ºC. The upper, clear phase was transferred into clean tubes and stored at –80ºC for subsequent use for Western blot analysis.

### 4.4.2 Western Blot Analysis

Protein concentrations in each sample were detected by using a BCA (Bicinchoninic Acid) kit (Pierce Chemicals, Rockford, IL). The colourimetric reactions were measured by using a DU-64 UV-spectrophotometer (Beckman Coulter, Mississuaga, ON, Canada) using a wavelength of 595 nm. An aliquot (15µg) of the protein sample was prepared by dilution, after which loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, 0.025% SDS) was added to each sample.The mixture was then boiled for 5minutes.

For gel preparation, glass plates were cleaned using 70% ethanol. The Western blot apparatus was set up as follows: running gel (7% for E-cadherin, 15% for Twist and Actin) was poured between the glass plates and covered with ethanol (100%). After the gel had solidified, the ethanol covering the gel was removed. The stacking gel was poured on top and a comb (50µl/well) was inserted. After the stacking gel had solidified, running buffer was added until the stacking gel was covered. The comb was then removed and the gel washed with distilled water.

The samples prepared beforehand were loaded into each of the wells. The unit was connected to the electric power pack and run at 100V for about 1.5 hours. Approximately, 15 minutes before the gel was complete, 6 pieces of filter paper (8cm x 5cm) and 1 piece of nitrocellulose membrane (NC membrane) (Hybond-P, Amersham Pharmacia Biotech, Oakville, Canada) were cut to match the size of the running gel. The NC membrane and filter paper were soaked in Transfer Buffer (0.3% Tris, 1.44% glycine, 80% water and 20% methanol) for 10 minutes to remove any air pockets. After the gel had finished running, the stacking gel was removed from the Western blot apparatus. The running gel, the filter paper and NC membrane were layered in the following order: the black side of the cassette, three pieces of filter paper,

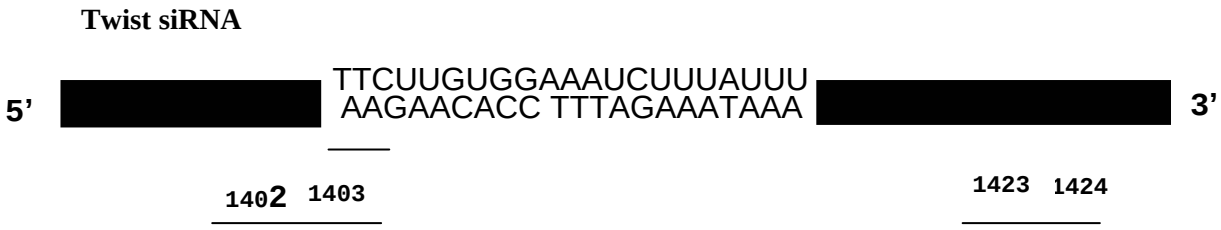
gel, NC membrane, three pieces of filter paper, and finally the white side of the cassette. The cassette was then placed into the transfer apparatus. Transfer buffer was then added to the apparatus until the whole transfer cassette was submerged. The transfer apparatus was connected to the electric power unit and run under constant current (500 mA) for 1 hour.

After the transfer of the electrophoresed proteins onto the NC membrane was complete, the membrane was removed and incubated with blocking buffer (5% skim milk in Tris buffered saline (TBS) (50mMTris, 0.15M NaCl) for 30 minutes. The blocking reagent reduces the likelihood of detecting non-specific bands on the Western blot. Then the membrane was incubated with a monoclonal antibody directed against E-cadherin (Transduction Laboratory, Lexington, KY), β-actin (Transduction Laboratory), or Twist (Santa Cruz Biotechnology, Santa Cruz, CA), each diluted to a final volume (1:500) in blocking buffer. The blots were incubated with these primary antibodies overnight at 4ºC. The membrane was then washed three times in TBS containing 0.1% (v/v) Tween 20 for a total of 30 minutes on a rocking platform. The membrane was then incubated with a mouse monoclonal secondary antibody isotype-matched for the primary antibodies (diluted 1:2,000 in blocking buffer) for 1 hour. The membrane was subsequently placed in washing buffer for 10 minutes on a rocking platform. The enhanced chemiluminescence (ECL) system (Amersham Phamacia Biotech, Oakville, Canada) was then added to detect antibody bound to antigen. The reaction was allowed to proceed for 5 minutes, after which the membrane was then wrapped in plastic wrap and exposed to X-ray film for an appropriate period of time (ranging from 2 to 30 minutes). The resultant autoradiogram was scanned using an LKB laser densitometer. The absorbance value obtained for E-cadherin and Twist in each of the cell lysates was normalized relative to the corresponding β-actin absorbance value.

Figure 2. Design of human Twist siRNA. Twist siRNA was designed according to a well-established protocol. The designed Twist siRNA sequence is located downstream of the startcodon, at position 1403bp to 1423bp with a length of 21bp. The style of the sequence is (N19)TT. The percentage of GC content in the siRNA sequence is 25%. Finally, tt was been confirmed that the sequence of the siRNA is unique for the human Twist gene by the BLAST.

1. To limit the percentage of GC content in the siRNA sequence within 30—70%**.**

2. To be the sequence of 23-nucleotide such as AA (N19) TT, or AA



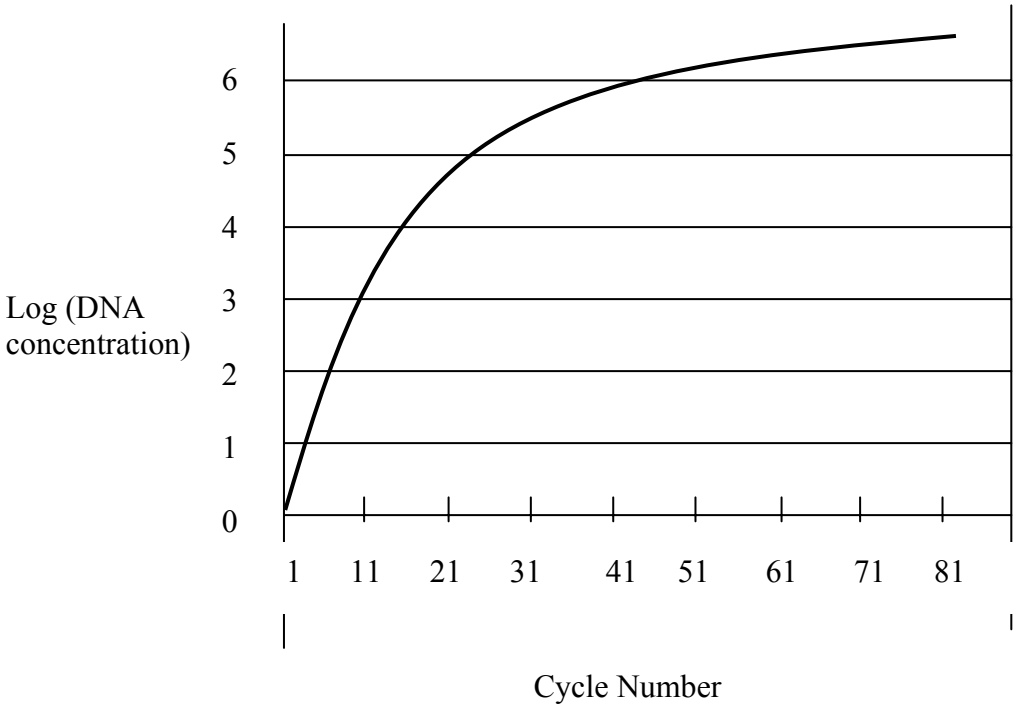

4. The sequence of siRNA is targeted to only one gene in genome

3. To be 50-100 bases downstream of the startcodon

Table 1. Primer Design and Optimized PCR Conditions

| Gene | | |
|---|---|---|
| GAPDH | Primer sequences | Forward primer:<br>**5'-CCCCAATGTGTCCGTCGTG-3'**<br>Reverse primer:<br>**5'-GCCTGCTTCACCACCTTCT-3'** |
| | PCR Conditions | Denaturing temp 94° C for 1 min ,<br>Annealing temp 55° C for 45 sec,<br>Extension temp 72° C for 1.5 min. |
| | Cycles | 20 cycles |
| E-Cad | Primer sequences | Forward primer:<br>**5'-TGGATGTGCTGGATGTGAAT-3'**<br>Reverse primer**:**<br>**5'-ACCCACCTCTAAGGCCATCT-3'** |
| | PCR Conditions | Denaturing temp 94° C for 1 min,<br>Annealing temp 57° C for 1 min,<br>Extension temp 72° C for 1.5 min. |
| | Cycles | 30 cycles |
| N-Cad | Primer sequences | Forward primer**:**<br>**5'-ACAGTGGCCACCTACAAAGG-3'**<br>Reverse Primer:<br>**5'-TGATCCCTCAGGAACTGTCC-3'** |
| | PCR Conditions | Denaturing temp 95ºC 30 sec,<br>Annealing temp 58ºC 45 sec,<br>Extension temp 72ºC 1 min. |
| | Cycles | 24 cycles |
| Twist | Primer sequences | Forward primer:<br>**5'-AGTCCGAGTCTTACGAGGA-3'**<br>Reverse primer**:**<br>**5'-GCAGAGGATGTGAGGATGGT-3'** |
| | PCR Conditions | Denaturing temp 94ºC 1 min,<br>Annealing temp 58.5ºC 1 min,<br>Extension temp 72ºC 1.5 min. |
| | Cycles | 30 cycles |

Figure 3. A saturation curve of RT-PCR product. The dotted line presents the optimized cycle number to be selected.



## 5. Statistical Analysis

The absorbance values obtained from the ethidium bromide stained gels containing the PCR products and the autoradiograms generated by Western blotting were obtained by using GraphPad Prism 4 computer software (GraphPad, San Diego, CA, USA). The results are presented as the mean relative absorbance obtained from 2 different experiments. Error bars represent standard deviation across 2 experiments by using function of STDEV. Because the number of replicates of each experiment is 2, significant differences between the means can not be determined.

# 6. Results

## 6.1 Time-dependent Effects of Twist siRNA on Twist and E-cadherin mRNA and Protein Expression Levels in BeWo Cells

A 5-fold decrease in Twist mRNA was detected in BeWo cells after 24 h of transfection with siRNA specific for this transcription factor compared with 24 h control (Fig. 4, A and C). Levels of the Twist mRNA transcript continued to decrease until the termination of these studies at 48 h (Fig. 4, B and C). In contrast, there was a concomitant increase in E-cadherin mRNA levels in these cell cultures over the same time period (Fig. 4, A and C). Transfection of BeWo cells with the control siRNA had no great effect on Twist and E-cadherin mRNA levels over the same time points (Fig. 4, A-C).

In agreement with the preceding RT-PCR data, a Twist protein species of 35kDa was detected in all of the BeWo choriocarcinoma cell cultures (Fig. 5, A and C). The levels of this protein species remained relatively constant in BeWo cells transfected with the control siRNA at all of the time points examined in these studies (Fig. 5, A and C). In contrast, a decrease in Twist protein expression levels was detected in BeWo cells transfected with Twist siRNA after 24 h (Fig. 5, A and C). Levels of the Twist protein species remained low until the termination of these studies of 48 h (Fig. 5, A and C).

An E-cadherin protein species of 120kDa was detected in all of the BeWo choriocarcinoma cell cultures (Fig. 6, A and C). The levels of this protein species remained relatively constant in BeWo cells transfected with the control siRNA at all of the time points examined (Fig. 6, A and C). In contrast, an increase in E-cadherin proteins expression level was detected in BeWo cells after 24 h of transfection with Twist siRNA (Fig. 6, A and C). E-cadherin expression levels remained going up until the termination of these studies at 48 h (Fig. 6, A and C).

In this set of experiments, I have determined that decreased Twist expression in BeWo cells resulted in a concomitant increase in E-cadherin mRNA and protein expression levels in these cells over time in culture. I then went on to determine whether loss of Twist expression would alter N-cadherin expression levels in cultures of highly invasive EVTs.

## 6.2 Concentration-dependent Effects of Twist siRNA on Twist and N-cadherin mRNA Levels in Cultures of EVTs.

Initial studies demonstrated that Twist and N-cadherin mRNA transcripts were present in cultures of EVTs (Fig. 7 B). A decrease in Twist mRNA was detected in EVTs after 24 h of transfection with Twist siRNA (from 1μg to 4μg) and Transmessenger (volumes ranging from 5μl to 20μl) (Fig. 7 B and C). The lowest levels of Twist mRNA transcript were obtained in EVTs following transfection with a combination of 2μg siRNA and 10μl Transmessenger (Fig. 7 B and C). There was a concomitant decrease in N-cadherin mRNA levels in these cell cultures after 24 h transfection cultured under the corresponding experimental conditions (Fig. 7 A and C).

## 6.3 Time-dependent Effects of Twist siRNA on N-cadherin and Twist mRNA Levels in EVTs.

A decrease in Twist mRNA levels was first detected in these cell cultures after 24 h of transfection with Twist siRNA (Fig. 8 A and C). The level of the Twist mRNA transcript continued to decline until the termination of these studies at 72 h (Fig. 8 B and C) Similarly, there was a marked decrease in N-cadherin mRNA levels after 24 h of transfection with Twist siRNA with decrease in the levels of this mRNA transcript being observed at the termination of these studies (Fig. 8 A and C). However, in the non-sense control experiments, there also occurred decreased mRNA level of N-cadherin after 48 h and 72 h of transfection, but Twist mRNA level was more constant except that after 72 h of transfection. By comparasion of N-cadherin mRNA level of experiments with Twist siRNA transfection and non-sense control siRNA transfection, it is regarded that the number of measuring N-cadherin mRNA is not large enough to provide a statistically more accurate amount of N-cadherin mRNA level. A larger experiment scale would be helpful to further confirm the result.

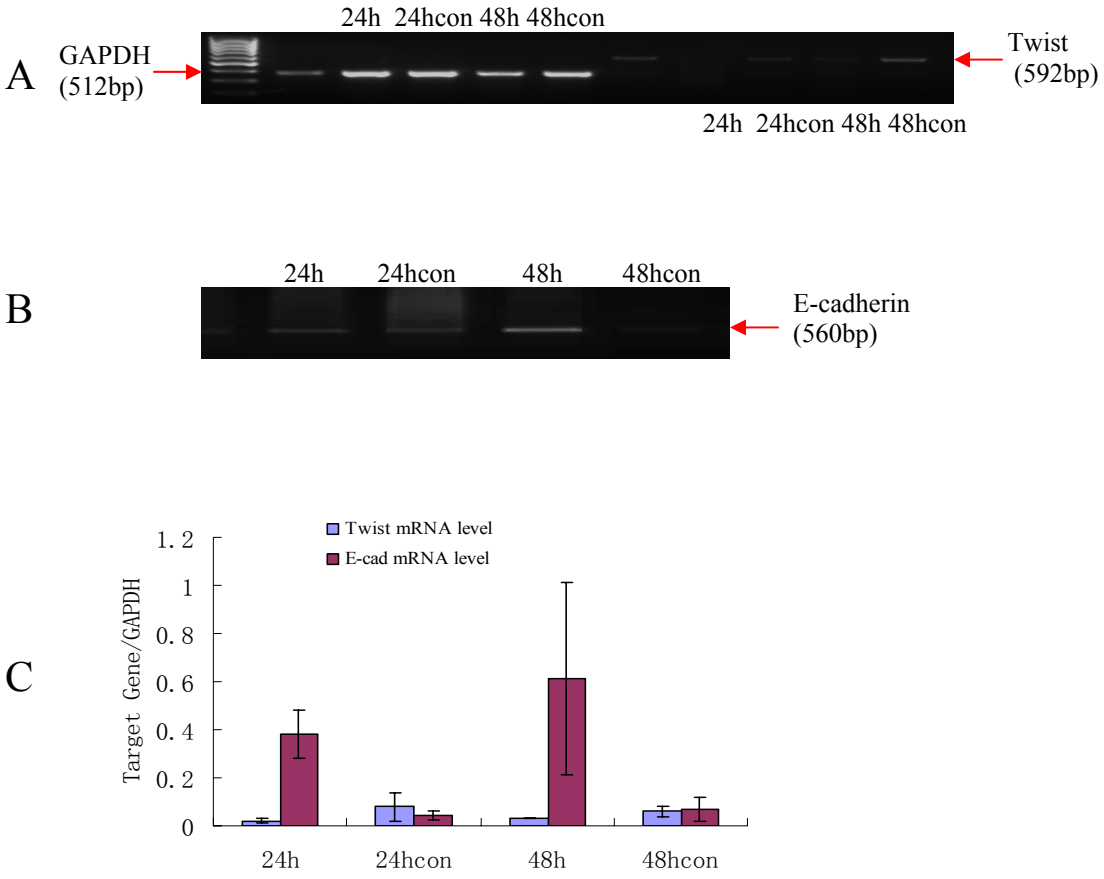



Figure 4. Twist and E-cadherin mRNA levels in BeWo cells following transfection with Twist siRNA. (A) RT-PCR analysis of Twist mRNA and GAPDH mRNA in BeWo cells following transfection with Twist siRNA for 24 and 48 h (lanes 24h and 48h) or nonsense siRNA (lanes 24hcon and 48hcon). (B) RT-PCR analysis of E-cadherin mRNA levels in BeWo cells following transfection with Twist siRNA for 24 or 48 h (lanes 24h and 48h), or nonsense siRNA transfection (lanes 24hcon and 48hcon). (C) The absorbance values obtained for E-cadherin or Twist were normalized to these of the corresponding GAPDH mRNA. The results derived from this analysis, as well as those from another study are presented (n=2) in the bar graph.

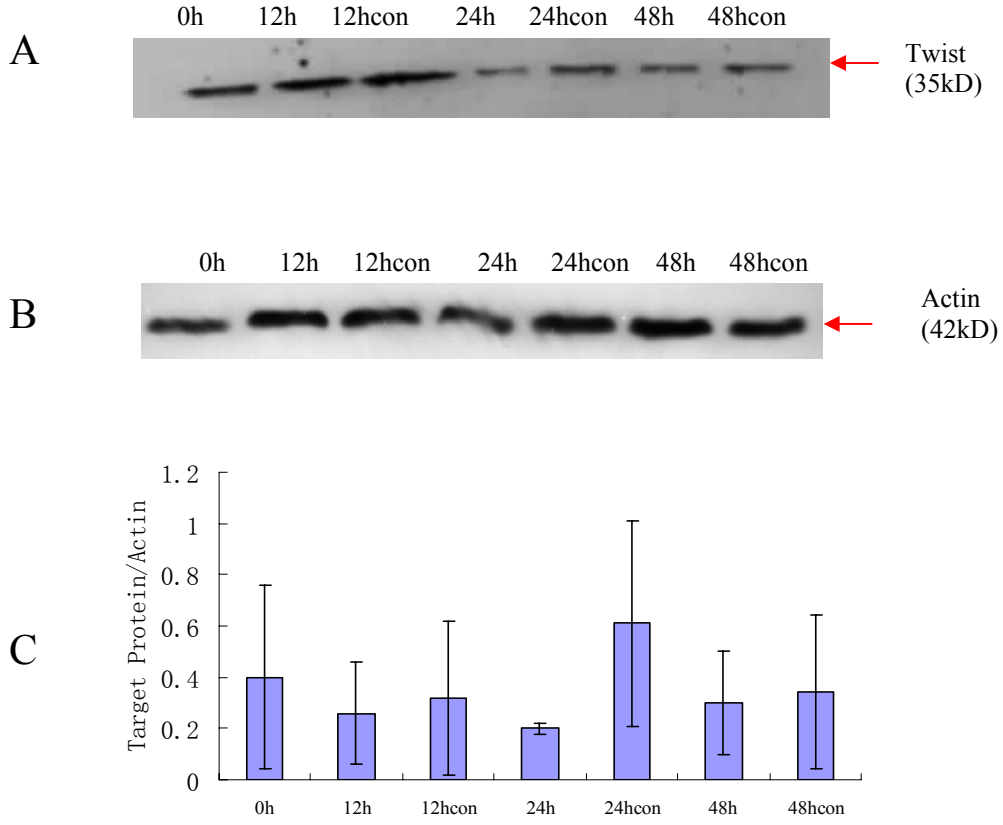



Figure 5. Twist protein levels in BeWo cells following transfection with Twist or control siRNA. (A) Western blot analysis of Twist protein expression levels in BeWo cells following transfection with Twist siRNA (lanes 0h, 12h, 24h and 48h) or nonsense siRNA (lanes 12hcon, 24hcon and 48hcon) for 0, 12, 24 and 48hour respectively. (B) Western blot analysis of β-actin protein expression levels in BeWo cells following transfection with Twist siRNA (lanes 0h, 12h, 24h and 48h) or nonsense siRNA (lanes 12hcon, 24hcon and 48hcon) after 0, 12, 24 and 48hour respectively. (C) The Amersham ECL system was used to detect antibody bound to antigen. The resultant autoradiogram was scanned and the absorbance values obtained for Twist are normalized to those of β-actin. The results derived from this analysis as well as those from another study (n=2) are presented in the bar graph.

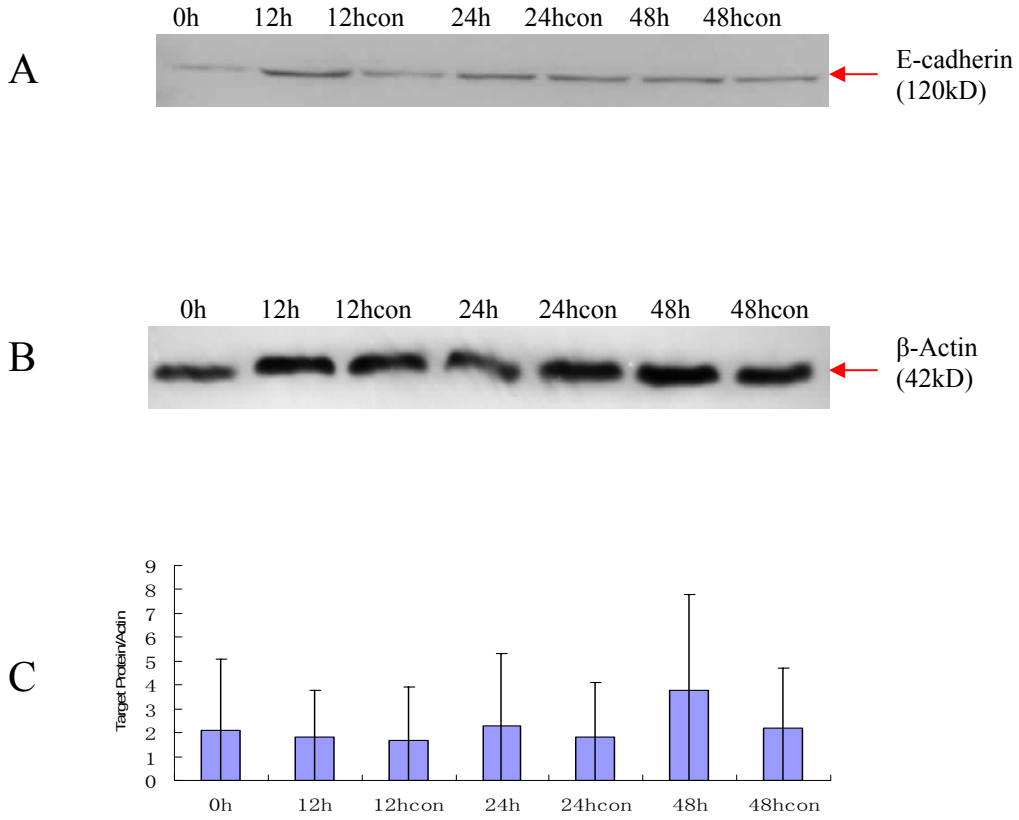



Figure 6. E-cadherin protein levels in BeWo cells following transfection with Twist or control siRNA. (A) Western blot analysis of E-cadherin protein in BeWo cells following transfection with Twist siRNA (lanes 0h, 12h, 24h and 48h), or nonsense siRNA (lanes 12hcon, 24hcon and 48hcon) for 0, 12, 24 or 48 h, respectively. (B) Western blot analysis of β-actin in BeWo cells following transfection with Twist (lanes 0h, 12h, 24h and 48h) or nonsense siRNA (lanes 12hcon, 24hcon and 48hcon) for 0, 12, 24 and 48 h, respectively. (C) The absorbance values obtained for E-cadherin were normalized to these of β-actin protein expression levels in corresponding cell cultures. The results derived from this analysis, as well as those from another study (n=2) are presented in the bar graph.

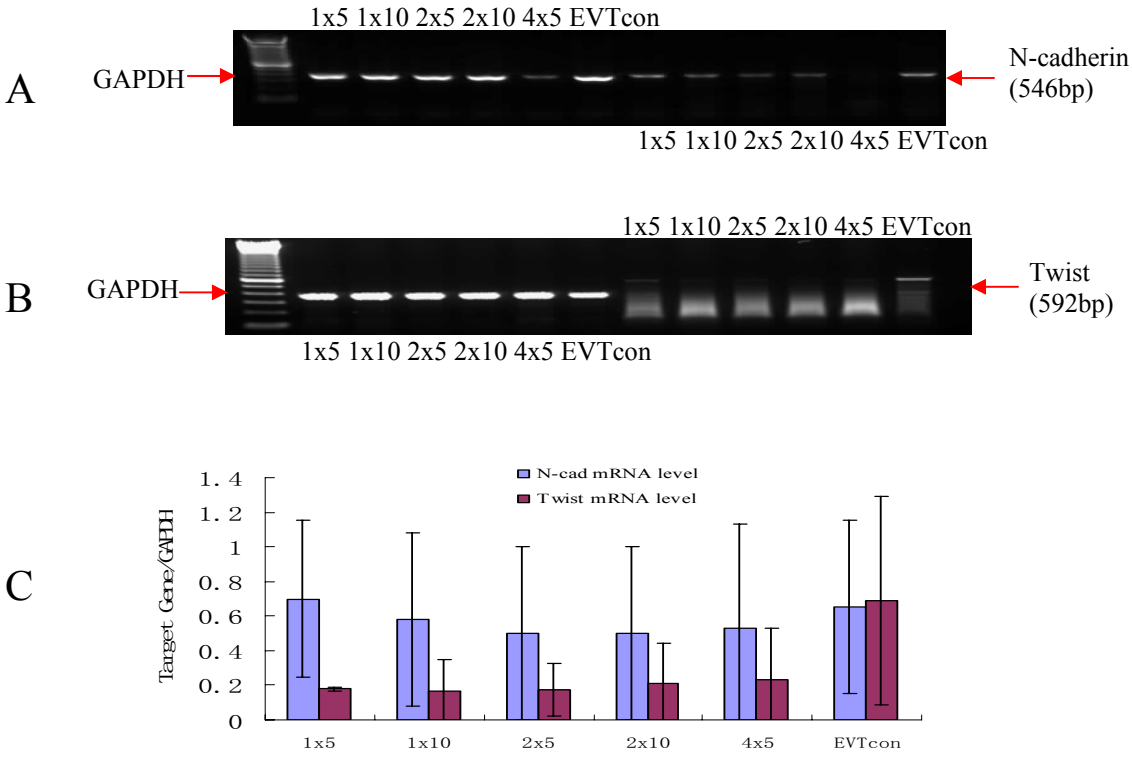
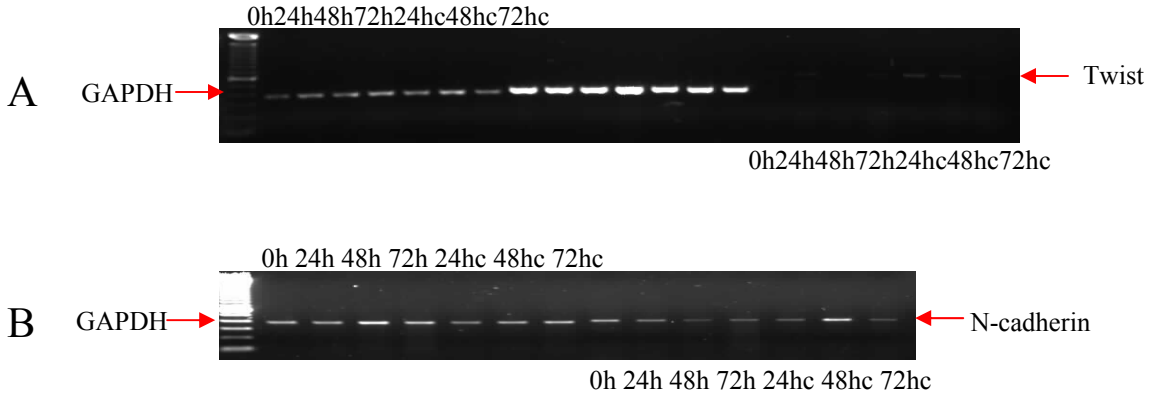



Figure 7. Dose-dependent effects of Twist siRNA on N-cadherin and Twist mRNA levels in EVTs. (A) RT-PCR analysis of N-cadherin level and GAPDH mRNA levels in EVTs following transfection with Twist siRNA 1μg siRNA plus 5μl Transmessenger treatment (lane 1x5), 1μg siRNA plus 10μl Transmessenger (lane 1x10), 2μg siRNA plus 10μl Transmessenger (lane 2x5), 2μg siRNA plus 20μl Transmessenger (lane 2x10), 4μg siRNA plus 20μl Transmessenger (lane 4x5) and 0μg siRNA plus 0 Transmessenger (lane EVT control) for 24 h. (B) RT-PCR analysis of Twist (592bp) and GAPDH mRNA in EVTs following transfection with Twist siRNA of 1μg siRNA plus 5μl Transmessenger treatment (lane 1x5), 1μg siRNA plus 10μl Transmessenger (lane 1x10), 2μg siRNA plus 10μl Transmessenger (lane 2x5), 2μg siRNA plus 20μl Transmessenger (lane 2x10), 4μg siRNA plus 20μl Transmessenger (lane 4x5) and 0μg siRNA plus 0 Transmessenger (lane EVT control) for 24 h respectively. (C) The absorbance ratios obtained for N-cadherin or Twist mRNA levels were normalized to the corresponding GAPDH. The results derived from this analysis, as well as those of another study (n=2) are presented in the bar graph.



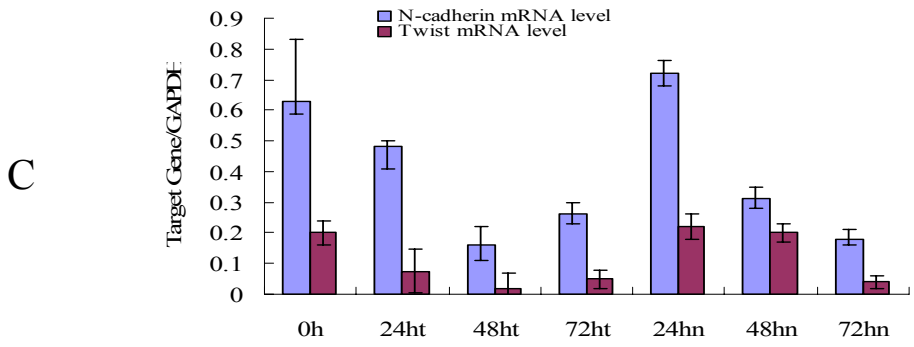




Figure 8. Time-dependent effects of Twist siRNA on N-cadherin and Twist mRNA in EVTs. (A) RT-PCR analysis of Twist and GAPDH mRNA in EVTs following transfection with Twist siRNA 2μg (lanes 0h, 24h, 48h and 72h) or nonsense siRNA (lanes 0hc, 24hc, 48hc and 72hc) after 0, 24, 48 and 72 h respectively. (B) RT-PCR analysis of N-cadherin and GAPDH mRNA level in EVTs following transfection with Twist siRNA 2μg (lanes 0h, 24h, 48h and 72h) or nonsense siRNA (lanes 0hc, 24hc, 48hc and 72hc) after 0, 24, 48 and 72 h respectively. (C) The absorbance values for obtained N-cadherin or Twist mRNA levels were normalized to the corresponding GAPDH. The results derived from this analysis, as well as those from another study (n=2) are presented in the bar graph.

# 7. Discussion

## 7.1 Twist Regulation of E-cadherin and N-cadherin in Human Trophoblastic Cells *in vitro*

In this study, I used an siRNA transfection strategy to decrease Twist gene expression in BeWo cells and EVTs. In BeWo cells, a decrease in Twist mRNA and protein expression levels was concomitant with an increase in E-cadherin mRNA and protein expression levels. These observations are in agreement with previous studies using a wide variety of cancer cells in which Twist induces EMT through its regulation of E-cadherin mRNA and protein expression levels (Yang et al., 2004; Kwok et al., 2005; Yuen et al., 2007; Vesuna et al., 2008).

The mechanism by which decreased Twist expression results in a concomitant increase in E-cadherin mRNA and protein expression levels in these trophoblastic cells was not investigated in this study. However, Twist has been shown to be a transcription repressor of E-cadherin gene expression in embryonic cells and breast cancer cells via direct interactions with the E-Box present in the promoter region of this gene in humans (Yang et al., 2004).

I also used this siRNA transfection strategy to decrease Twist gene expression in EVTs. A decrease in Twist mRNA and protein expression levels in these trophoblastic cells was found to be concomitant with a decrease in N-cadherin mRNA in these cells. The decrease in the levels of the mRNA transcript encoding N-cadherin in these cells are in agreement with previous studies, in which N-cadherin mRNA levels were up-regulated in gastric and prostatic carcinoma and this was correlated with an increase in Twist expression levels (Rosivatz et al., 2002; Alexander et al., 2006; Yang et al., 2007). My studies using invasive trophoblastic cells suggest that Twist may also be a regulator of N-cadherin in human EVTs, a highly invasive subpopulation of cells present in the placenta. In previous studies, Ectopic expression of Twist was performed by transfecting a mammalian expression vector containing the N-cadherin gene into EVTs, which confirmed its regulatory effects on N-cadherin mRNA and protein levels in these cells.

The mechanism by which Twist regulates N-cadherin was not investigated. However, as the N-cadherin gene contains an E-Box in intron1, I propose that Twist might exert its

regulation on N-cadherin also through this E-Box. To test this hypothysis, the luciferase system assay could be employed, in which the transcription efficiency of N-cadherin would be measured with an E-Box mutation.

Human trophoblasts are distinct epithelial cell types that differentiate during embryonic development (Selwood et al., 2006). These epithelial cells can either undergo proliferation and adopt a highly invasive phenotype or undergo terminal differentiation and fusion to form a multinucleated mitotically inactive syncytium (Bischof et al., 2005). These development processes are associated with a cellular phenomenon commonly referred to as "cadherin switching", during which E-cadherin is decreased and N-cadherin is increased (Yang et al., 2004).

## 7.2 Twist is Involved in the Trophoblast Differentiation Process

In particular, expression of E-cadherin, N-cadherin, cadherin-11, and VE-cadherin are associated with the differentiation of the trophoblasts along either the invasive and/or non-invasive pathways. E-cadherin expression is high in villus cytotrophoblasts and decreases with either the development of an invasive phenotype and with the terminal differentiation and fusion of these mononucleate cells to form multinucleated syncytia (Knofler et al., 2004; Batistatou et al., 2007). In support of a role for Twist in regulating E-cadherin in this developmental process, Twist mRNA and protein levels were low in the poorly invasive BeWo chorionic carcinoma cells, a trophoblast model system used to study the morphological and biochemical differentiation of mononucleate cytotrophoblasts along the non-invasive pathway. Interestingly, higher Twist mRNA levels in these cells have also been associated with increased levels of cadherin-11 in gastric carcinoma. Cadherin-11 is up-regulated during the terminal differentiation and fusing of human trophoblastic cells *in vitro* and is preferentially expressed in the syncytial trophoblast layer of first trimester chorionic villi in vivo (Getsios et al., 2003). In view of these observations it is tempting to speculate that Twist may be responsible, at least in part, for the down-regulation of E-cadherin and the concomitant increase in cadherin-11 during this development process.

E-cadherin mRNA transcripts have not been detected in cultures of highly invasive EVTs (Floridon et al., 2000). Instead N-cadherin is one of the predominant cadherin subtypes present in these primary cell cultures. N-cadherin is also preferentially expressed in metastatic human carcinoma cells. Elevated levels of Twist have also been detected in these cancer cells, suggesting that there is a direct correlation between N-cadherin and Twist in the development of an invasive phenotype either via up-regulating N-cadherin and/or down-regulating E-cadherin, another characteristic of epithelial cells undergoing neoplastic transformation and the progression of cancer to the later stages of the disease state (Yang et al., 2006).

Loss- or gain- of function studies have subsequently determined a critical role for Twist in the regulation of N-cadherin expression in human carcinoma cells *in vitro* and in vivo (Yang et al., 2007). The ability of decreased Twist expression levels in EVTs to result in a concomitant decrease in N-cadherin mRNA levels in these cells provides further evidence that human trophoblastic cells utilize the same molecular mechanism used by human cancer cells as they develop an invasive phenotype (Yang et al., 2007). However, unlike cancer cells, trophoblast invasion is a highly ordered and highly regulated developmental process (Yang et al., 2007). Thus, human trophoblastic cells may serve as a useful model system in which to further elucidate the molecular pathway(s) underlying cadherin-mediated, cell fate determination.

Twist is a universal transcription factor (Castanon et al., 2002). It is able to regulate a wide variety of genes, many of which are also regulated during trophoblast differentiation along either the non-invasive or invasive pathway (Castanon et al., 2002). Further studies will be required to determine whether these signaling pathways operate independently, in parallel, or as a network during the differentiation of human trophoblasts or /and the formation and organization of the human placenta.

Other factors identified in the placenta have been shown to regulate the expression and function of E-cadherin and N-cadherin. For example, Snail down regulates E-cadherin in ovarian carcinoma cells (Elloul et al., 2006). Snail also up-regulates N-cadherin in oral squamous carcinoma cells (Takkunen et al., 2006). Loss- or gain- of function in the trophoblast cells has shown that N-cadherin is critical for trophoblast differentiation (Yang et al., 2001). This CAM is also regulated in a spatio-temporal manner during embryonic

development. In my study, I have demonstrated that altered expression of Twist results in the differential regulation of E-cadherin and N-cadherin expression in trophoblastic cells. To date, the hierarchy of molecular relationships, if any, that would link these pathways remains to be elucidated. Collectively, these observations indicate that Twist is involved in the formation and development of human placenta through its regulation of E-cadherin, N-cadherin and other related genes. Although the data presented in this study provide useful insight into the potential regulatary role of Twist in the regulation of N-cadherin and E-cadherin in human trophoblastic cells, it is not possible to determine whether these effects are direct or indirect. Furthermore, it is not possible to determine whether alterations in Twist result in constant changes in the invasive capacity of trophoblastic cells, and whether Twist is involved in loss of E-cadherin during differentiation of trophoblasts along the non-invasive pathway.

## 7.3 Twist Involvment in Preeclampsia

Preeclampsia is a pregnancy disorder characterized by insufficient trophoblast differentiation (Cnossen et al., 2006; Batistatou et al., 2007). Its exact molecular mechanism has yet to be elucidated. Cytotrophoblast cells from preeclampsia cases often show aberrant expression patterns of cadherin subtypes (Cheng et al., 1999). In particular, expression levels/patterns of E-cadherin in cytotrophoblasts isolated from preeclamptic placental tissues or in cytotrophoblasts cultured under $O_2$ tension are aberrantly up-regulated (Myatt et al., 2004). In view of my observations, it is tempting to speculate that Twist expression levels will also be altered in these cells *in vivo* and *in vitro*. Alternatively, placenta accreta, increta choriocarcinoma and other persistent trophoblastic diseases are characterized by abnormally deep invasion of trophoblasts into the underlying maternal tissues (Cnossen et al., 2006; Batistatou et al., 2007). Not surprisingly, aberrant cadherin expression has been detected in the implantation sites of these pregnancy disorders (Lessey et al., 1997; Batistatou et al., 2007; Belchschmidt et al., 2008). Thus, Twist may serve as a novel target gene for the development of novel cell-based therapies for the treatment of preeclampsia and other disorders of pregnancy that are associated with aberrant trophoblast invasion (Fig. 10).

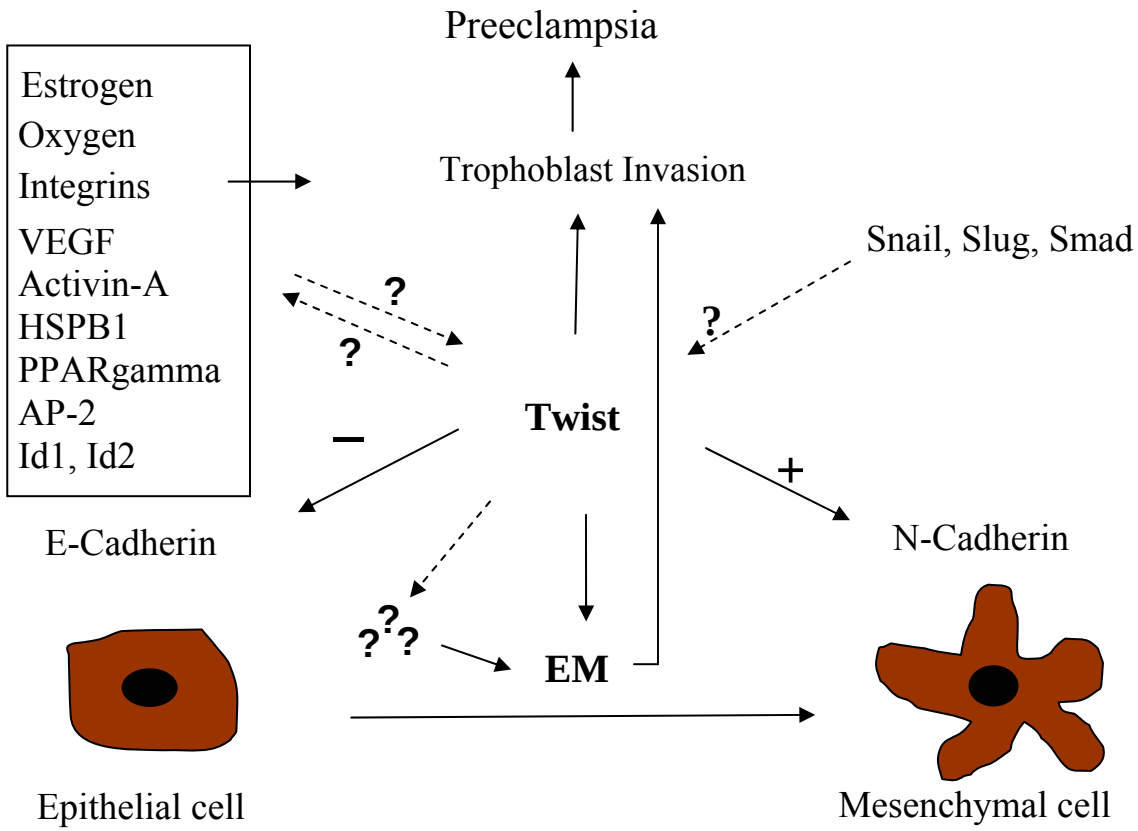



Figure 9. A schematic diagram illustrating a proposed role for Twist in cadherin-mediated trophoblast differentiation. Twist is capable of down-regulating E-Cadherin and up-regulating N-Cadherin during EMT. Twist has the potential to regulate other factors involved in the EMT process. All of these could subsequently control trophoblast differentiation, which is abnormally repressed during preeclampsia and high in persistent trophoblastic disease. Twist and some other factors including estrogen, oxygen, integrins, VEGF and Activin-A also regulate the trophoblast differentiation. Snail, Slug and Smad (Sma and Mad related proteins) are also transcriptional factors involved in embryonic development, and therefore could serve as downstream/upstream candidates for trophoblast differentiation.

# 8. Summary

My studies demonstrate by using an siRNA knockdown gene strategy, that Twist is a key regulator of mRNA and protein levels of E-cadherin and N-cadherin in human trophoblastic cells *in vitro*. Loss of Twist expression resulted in an increase in E-cadherin expression in poorly invasive BeWo chorionic charcinoma cells and decreased N-cadherin expression levels in highly invasive EVTs. As the differential expression of these two cadherins is associated with the invasive phenotype of other epithelial cells during EMT, and Twist is a key regulator of this developmental process, my studies provide further evidence that human trophoblastic cells employ similar molecular mechanisms to those adopted by metastatic cancer cells. Further studies will examine whether alterations in Twist mRNA and protein expression levels, using gain-of-function studies, alter the invasive capacity of human trophoblastic cells *in vitro*. This would be achieved by the expression of exogenous Twist using a mammalian expression vector. It is anticipated that this gain-of-Twist function would promote the invasive capacity of these two types of trophoblastic cells. To better define the biological significance of Twist in human trophoblast differentiation *in vivo*, it will be necessary to examine the spatio-temporal expression of this transcription factor in human placental tissues under normal and pathological conditions by using immunohistochemical analysis.

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