

**CADHERIN-LINKED MOLECULAR MECHANISMS GOVERNING THE  
TERMINAL DIFFERENTIATION OF HUMAN TROPHOBLASTIC CELLS IN  
VITRO**

by

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## **Abstract**

**Background:** The formation of the multinucleated syncytial trophoblast of the human placenta is a critical step in pregnancy, which is prone to failure. In these studies, I have examined the role of TWIST, a transcription factor identified as a key repressor of E-cad expression in normal and cancer cells of diverse origins, in the differentiation of human trophoblastic cells *in vitro*. The invasion of extravillous cytotrophoblasts (EVTs) into the underlying maternal tissues and vasculature is a key step in human placentation. The molecular mechanisms underlying the development of the invasive phenotype of EVTs include many of those first identified as having a role in cancer cell metastasis. In view of these observations, I have examined the expression, regulation, and function of Twist, Runx2 and N-cad in human trophoblastic cells *in vitro*.

**Materials and Methods:** Gain or loss-of-function studies were then performed to determine the role of Twist in terminal differentiation and fusion in these cells. The presence of multinucleated syncytium was confirmed by indirect immunofluorescence. Concentration- and time-dependent studies were performed to determine whether interleukin (IL)-1 $\beta$  and transforming growth factor (TGF)- $\beta$ 1 regulate Twist and Runx2 mRNA and protein levels in EVTs. Next, a siRNA strategy was employed to determine the role of Twist, Runx2 and N-cad in HTR-8/SVneo EVT cells.

**Results:** Exogenous expression of Twist resulted in a continuous and progressive decrease in E-cad expression and the subsequent formation of syncytium in BeWo cells maintained under normal culture conditions. In contrast, siRNA specific for Twist inhibited the cAMP-mediated differentiation of these cells over time in culture. The cytokines, IL-1 $\beta$  and TGF- $\beta$ 1, respectively induced the differential up- and down-

regulation of Twist and Runx2 expression in primary cultures of EVT<sub>s</sub> in both a concentration and time-dependent manner. Use of a siRNA strategy demonstrated that a reduction in Twist, Runx2 or N-cad in HTR-8/SVneo cells concomitantly decreased the invasiveness of these cells.

**Conclusions:** Collectively, my findings demonstrate that TWIST is an upstream regulator of the E-CAD-mediated terminal differentiation and fusion of human trophoblastic cells *in vitro*. TWIST, RUNX2 and N-CAD are key molecules underlying the invasive capacity of EVT<sub>s</sub>.

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
BrdU	5-bromo-2'-deoxy-uridine
C	Carboxy
°C	Degrees centigrade
Ca <sup>2+</sup>	Calcium
CaCl <sub>2</sub>	Calcium chloride
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine 3',5'-monophosphate
cDNA	Complementary DNA
CP	Cytoplasmic domain
Cx	Connexin
DMEM	Dulbeco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
E <sub>2</sub>	Estradiol
EC	Extracellular subdomain
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetate
EMT	Epithelial mesenchymal transition

EVT	Extravillous cytotrophoblast
FGF	Fibroblast growth factor
FGFR	FGF receptor
g	Grams
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hours
HAV	Histidine-alanine-valine
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP-1	IGF binding protein-1
IL-1 $\beta$	Interleukin-1 $\beta$
IUGR	Intrauterine growth restriction
Kb	Kilo bases
kDA	Kilo daltons
LacZ	$\beta$ -galactosidase
LH	Luteinizing hormone
Min	Minutes
ml	Milliliter
mM	Millimolar
MF	Microfilaments
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type MMP



MW	Molecular weight
mRNA	Messenger RNA
$\mu$ l	Microliter
$\mu$ M	Micromolar
Nm	Nanomolar
NaCl	Sodium chloride
<i>P</i>	Probability
PA	Plasminogen activator
PAGE	Polyacrylimide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulfonyl fluoride
RNA	Ribonucleic acid
RER	Rough endoplasmic reticulum
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	Short interference RNA
SV40	Simian vacuolating virus-40
TGF- $\beta$ 1	Transforming growth factor- $\beta$ 1
TIMP	Tissue inhibitor of MMP
TM	Transmembrane domain

TBS	Tris buffered saline
tPA	Tissue-type PA
Tris-HCL	Tris (hydroxymethyl)-aminomethane-hydrochloric acid
TSP	Thrombospondin type-1 repeat
uPA	Urokinase-type PA
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -d-galactosidase

## CHAPTER 1: OVERVIEW

### 1.1: Introduction

The human placenta plays a key role in regulating the growth, development, and survival of the fetus during pregnancy. It is the site of transfer of respiratory gasses, nutrients and waste products between the maternal and fetal systems; it serves as a barrier against blood-borne pathogens and the maternal immune system; and it fulfills an endocrine role by secreting hormones, growth factors and other bioactive substances required for the establishment and maintenance of pregnancy. The establishment and outcome of a pregnancy are highly dependent on the interactions and functional cooperation between the trophoblast and uterus (Pijnenborg *et al.*, 1980; Aplin, 1991). Abnormal placental development is associated with clinical pathological conditions such as miscarriage, intrauterine growth restriction or preeclampsia (King and Loke, 1994; Benirschke and Kaufmann, 2000). Moreover, abnormal placental development associated with fetal aneuploidy contributes to early pregnancy loss (Salafia *et al.*, 1993; van Lijnschoten *et al.*, 1994). In a similar manner, abnormal placental structure has deleterious effects on the growth of the fetus (Krebs *et al.*, 1996; Macara *et al.*, 1996).

The trophoblast is an extraembryonic fetal tissue originating from the trophectoderm of the blastocyst. During placental development, three trophoblastic cell populations can be identified: cytotrophoblast stem cells and their derivative cell types: the syncytiotrophoblast and the extravillous cytotrophoblast (Hertig *et al.*, 1956; Denker, 1993). The multinucleated syncytial trophoblast is formed from underlying mitotically

active, mononucleate cytotrophoblasts, and its formation is a cellular process dependent upon on a precise series of membrane-mediated events (Douglas and King, 1990). The cadherins are cell adhesion molecules capable of mediating the terminal differentiation and fusion of human cytotrophoblast (Getsios *et al.*, 2000).

In order for a human placenta to develop and function properly, the embryonic trophoblastic cells must proliferate, differentiate and invade into the maternal endometrium (Aplin, 1991). Studies have shown the important roles played by members of the cadherin subtype in cell differentiation during cancer development and cancer cell invasion (Oka *et al.*, 1993; Hazan *et al.*, 2000). These findings allow us to identify the potential molecular mechanisms involved in trophoblast invasion, as the process of human trophoblast invasion utilizes similar molecular mechanisms as those of tumour cell invasion, albeit trophoblast invasion is a more tightly regulated, developmental process (Lala *et al.*, 2002). The acquisition of the invasive and metastatic phenotype, and the transformation of a cell, result from complex cellular processes that will most likely regulate the levels and actions of transcription factors that control the genetic program. Very often, the regulatory factors associated with tumourigenesis are required for early development of tissues, including epithelial-mesenchymal transition genes (Twist, Snail, Slug, TGF- $\beta$ ) and phenotypic genes (Runx transcription factors) (Morrison and Kimble, 2006).

Careful control of gene expression by transcription factors is important in maintaining the physiological levels of proteins needed for normal cell function. However, expression of transcription factors can become aberrant in cancer cells due to epigenetic changes in chromatin, chromosome translocation or mutations (Yang *et al.*, 2004; Blyth *et al.*, 2005).

The main objective of my studies was to better understand the role(s) of cadherins in placental development. I have examined transcription factors, known as TWIST and RUNX2 during the terminal differentiation of human trophoblastic cells. In particular, TWIST is known to be a key regulator of cadherin-mediated interactions (Rosivatz *et al.*, 2002; Vesuna *et al.*, 2008). The ability of the cadherins to regulate the terminal differentiation of human trophoblastic cells was subsequently examined.

In this chapter, the development of the human placenta will be described with emphasis on the terminal differentiation of human cytotrophoblasts, particularly the molecular and cellular mechanisms involving the terminal differentiation of mononucleate cytotrophoblasts into either syncytiotrophoblasts or extravillous cytotrophoblasts. The cell biology of the cadherin gene superfamily and the transcription factors, TWIST and RUNX2, will also be reviewed.

## **1.2: Human implantation and placentation**

### 1.2.1: Terminal differentiation of human cytotrophoblasts

The first step in human implantation involves apposition and attachment of trophoblastic cells to the surface epithelium of the endometrium. After this initial contact, the trophoblastic cells penetrate into the basement membrane of the maternal endometrium (Schlafke and Enders, 1975; Bentin-Ley *et al.*, 2000). These stages of development are crucial in the process of establishing a successful pregnancy. Upon implantation, the embryonic trophoblast consists of two distinct but inter-related

epithelial cell layers: an inner layer of mitotically active cytotrophoblasts, and the outer syncytial trophoblast (Hertig *et al.*, 1956) (Figure 1.1). Even though the trophoblastic cell subpopulation that is involved in the earlier stages of invasion of maternal tissue is still not clearly defined (Pijnenborg, 1990; Aplin, 2000), histological studies have shown that both the cytotrophoblasts and the syncytial trophoblast interact with the epithelial cells in the endometrium (Enders, 1976). During the third week after ovulation, these trophoblastic cells, after infiltration by a vascularized fetal mesenchyme, organize into mature chorionic villous structures. The chorionic villi are made up of a single layer of villous cytotrophoblast cells that rest on a basement membrane, a mesenchymal core containing fetal blood vessels, and an outer layer of syncytial trophoblasts that are in contact with the maternal endometrium and blood. The development and existence of the chorionic villi has been denoted as the hallmark of the human haemochorial placenta, whereby the fetal circulatory system is separated from the maternal blood cells throughout all stages of pregnancy by at least a single layer of trophoblastic cells, the syncytial trophoblast. This epithelial layer is the most important maternal-fetal barrier (Boyd and Hamilton, 1970, McGann *et al.*, 1994).

The structural and functional properties of the villous cytotrophoblasts and the multinucleated syncytial trophoblast are different. For instance, the endoplasmic reticulum of villous cytotrophoblasts is poorly developed and non-vacuolated though it contains relatively large mitochondria and numerous free ribosomes (Boyd and Hamilton, 1970; Contractor *et al.*, 1977).

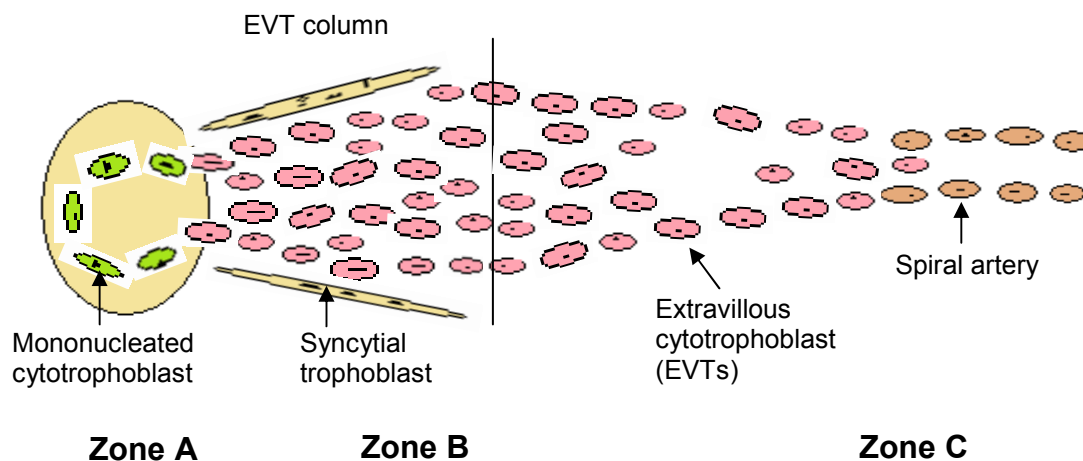
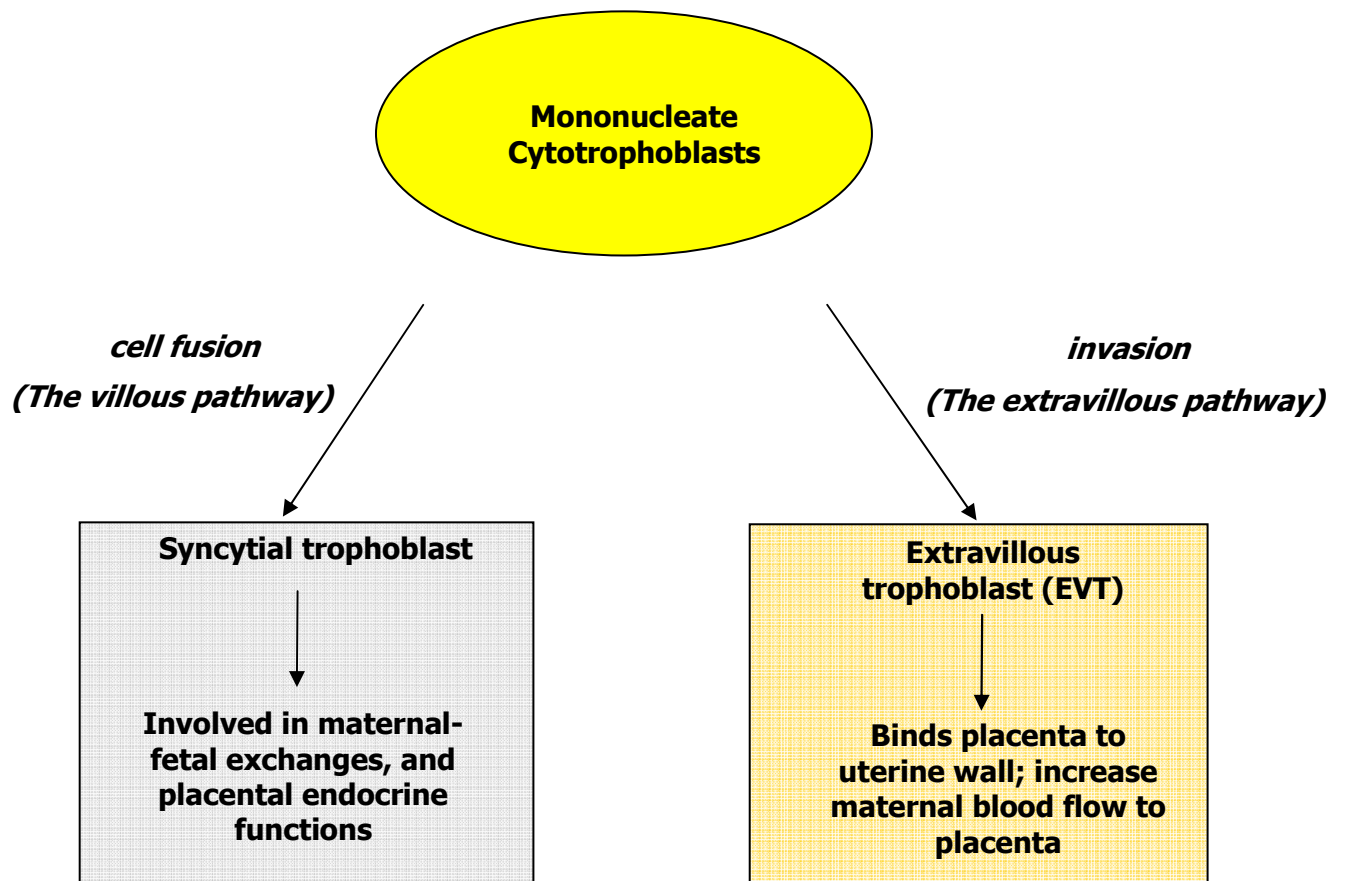


Figure 1.1. A) Schematic diagram representing human trophoblastic cell differentiation. Mononucleate cytotrophoblasts will undergo differentiation and fusion to form syncytial trophoblast or will proliferate and differentiate to become highly invasive extravillous cytotrophoblasts (EVTs). B) Schematic diagram representing chorionic villi. Zone A represents a floating villous consisting of mononuclear villous cytotrophoblasts entering the non-invasive pathway. Zone B represents an EVT column. Zone C represents the extravillous pathway.

Villous cytotrophoblasts seem likely to be the primary site of synthesis of several peptide hormones, such as activin, inhibin and gonadotropin-releasing hormone (Khodr and Siler-Khodr, 1980; Miyake *et al.*, 1982; Petraglia *et al.*, 1991; 1996). In contrast, syncytial trophoblast contains larger nuclei and has more developed organelles such as a vacuolated rough endoplasmic reticulum (RER) associated with protein synthesis, numerous mitochondria for steroid hormone biosynthesis, and Golgi apparatus and secretory vesicles for secretion functions (Boyd and Hamilton, 1970). The syncytial trophoblast is initially formed during implantation and then maintained as a kind of steady-state structure at the maternal-fetal interface throughout pregnancy (Huppertz, 1999). The syncytial trophoblast is a dynamic structure that forms the continuous outer layer of the human placenta. The majority of the biological functions of the human placenta, such as transporting gasses and nutrients from the maternal to the fetal circulation throughout pregnancy, is performed by the multinucleated syncytial trophoblast layer (Richard, 1961; Kliman *et al.*, 1986). Even though the mononucleate trophoblastic cells can produce human chorionic gonadotropin (hCG) at the earliest stages of pregnancy (Ohlsson *et al.*, 1989), the syncytial trophoblast becomes the major source of this and most other peptide and steroid hormones produced by the human placenta throughout pregnancy for placental growth and for maternal adaptation to pregnancy (Hoshina *et al.* 1982; Ringler and Strauss, 1990). Defects in the formation of the syncytial trophoblast are suspected to lead to several complications such as intrauterine growth restriction and pre-eclampsia (Lee *et al.*, 2001, Ishihara *et al.*, 2002).

In 1887, Langhans first suggested the cellular basis for the terminal differentiation in his morphological studies of the villous cytotrophoblasts in the human placenta (Boyd



and Hamilton, 1970). The human syncytial trophoblast is a terminally differentiated cell formed by post-mitotic fusion of the underlying villous cytotrophoblasts (Richard, 1961; Kliman *et al.*, 1986). Other investigations have proposed that the development of the multinucleated syncytial trophoblast occurs from nuclear duplication in the absence of cytokinesis, a process in which the cytoplasm of a single eukaryotic cell is divided to form two daughter cells (Sarto *et al.*, 1982). Functional studies using <sup>3</sup>H-thymidine incorporation in human trophoblastic cells as well as succeeding studies have shown that the villous cytotrophoblasts are mitotically active and that nuclear division is completely absent in the multinucleated syncytial trophoblast *in vivo* (Richard, 1961; Galton, 1962; Gerbie *et al.*, 1968). Ultrastructural analysis of the human term placenta demonstrated the presence of intercellular junctions within the syncytial trophoblast as well as within the boundary of direct contact between the villous cytotrophoblasts and the syncytial trophoblast. Furthermore, the junctional complexes within the syncytial trophoblast have been conceptualized as remnants of the cytotrophoblastic junctions that merged into the syncytial trophoblast cytoplasm following cellular fusion (Carter, 1964; Metz *et al.*, 1979; Metz and Weihe, 1980).

Recent evidence has suggested that envelope-like human endogenous retrovirus (HERV) protein known as syncytin-2 is a crucial mediator of the fusion process involved in the syncytialisation of trophoblasts. For example, real-time reverse transcription PCR and Western blot analyses in differentiating primary trophoblast cells have shown a direct correlation between mRNA and protein levels of Syncytin-2 and cell fusion. Furthermore, experiments with siRNA (small interfering RNA) transfected BeWo and

primary human trophoblast cells demonstrated an important diminution in the number of cell fusion processes upon repression of Syncytin-2 expression (Vargas *et al.*, 2009).

To date, the molecular and cellular mechanisms that mediate the formation of the syncytial trophoblast from the underlying villous cytotrophoblasts remain poorly characterized. Detailed studies on the molecular and cellular biology in the formation of the syncytial trophoblast are of critical importance for interpretation and treatment of pregnancy complications.

A successful human pregnancy depends upon mononucleate cytotrophoblasts entering one of the two distinct and mutually exclusive pathways. The villous cytotrophoblastic cells will proliferate and differentiate by fusion to form the outer syncytial trophoblast, as described above, or will enter the extravillous pathway to form highly invasive extravillous cytotrophoblasts (EVTs) (Bischof and Campana, 2000) (Figure 1.1).

In the extravillous pathway, cytotrophoblasts located within implanting chorionic villi differentiate into EVT's that invade deeply into the underlying maternal tissues and uterine vasculature, thus allowing an increased and controlled supply of blood flow to the placenta and ensuring an adequate supply of oxygen and nutrients to the developing fetus. This is a critical step in human pregnancy (Pijnenborg *et al.*, 1983 and 1994; Aplin, 1991).

Human EVT's can be divided into three populations, depending upon their molecular and morphological phenotypes and location within the extravillous compartment: intermediate cytotrophoblasts that proliferate are located near the villous basement membrane, interstitial cytotrophoblasts that will invade into the decidual stroma and

superficial myometrium, and endovascular cytotrophoblasts which will invade into the lumen of the spiral arteries (Pijnenborg *et al.*, 1981, 1983; Roberston *et al.*, 1986; Lala *et al.*, 2002; Bischof and Irminger-Finger, 2005). EVT's will undergo cell proliferation and break through the outer layer of the syncytial trophoblast to develop large cellular columns that stretch out into the maternal decidua (Enders, 1968; Muhlhauser *et al.*, 1993). Subpopulation(s) of EVT's will eventually detach from the tips of these cellular columns and invade into the decidual stroma and superficial myometrium as individual mononucleate cells and invade into the uterine arterial vasculature to replace the endothelial cells (Pijnenborg *et al.*, 1980, 1983). This cellular event is believed to remodel the smooth muscle and elastin layers of the arteries and the underlying endothelial cells in these blood vessels that subsequently allow for increased maternal blood flow to the placenta later in pregnancy, and to thereby ensure an adequate supply of oxygen and nutrients to the developing fetus (Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983).

The basic structure of the human placenta has become established at the end of the first trimester of pregnancy and all of the distinct trophoblastic cell subpopulations exist at the maternal-fetal interface (Boyd and Hamilton, 1970). Subsequently, trophoblastic cells continue to proliferate and differentiate, and promote placental growth until the end of pregnancy (Simpson *et al.*, 1992). Therefore, it is important to acquire a detailed understanding of the molecular mechanisms that regulate human trophoblast terminal differentiation, both fusion and invasion, during formation and organization of the human placenta.

## **1.2.2: Models used in studying human placentation**

### 1.2.2.1: Rodent models

Similarities have been noted between mouse and human in terms of placenta cell types (Carter, 2007) and genes controlling placental development (Rossant and Cross, 2001). The benefits of using the mouse as an experimental model include its short generation time. Other major benefits include the availability of embryonic stem cells, which help in gene targeting and the development of transgenic lines (Carter, 2007).

However, there are many differences between murine and human placentation; these include fewer placental hormones and a different mode of implantation in the mouse (Carter, 2007). More significantly, the transformation of uterine arteries in mice depends on maternal factors, such as the endothelium, rather than on trophoblasts and there is also limited trophoblast invasion in the mouse (Redline and Lu, 1989; Adamson *et al.*, 2002; Pijnenborg, 2006). It is important to bear in mind that there are still other differences between mouse and human placenta that need to be addressed, such as the labyrinthine rather than villous structure of the exchange area in the mouse and the presence of three layers of trophoblast in the interhaemal membrane (Carter, 2007). Therefore, the mouse is considered to be a less than ideal experimental model for studies of trophoblast invasion and vascular remodelling, in relation to the situation in humans.

#### 1.2.2.2: Non-human primate models

Studies have shown similarities among humans, baboons and macaques in terms of how the spiral arteries are invaded and transformed (Blankenship and Enders, 2003). Importantly, as in humans, the placenta of Old World monkeys (Cercopithecidae), e.g. baboon and macaque, are villous and haemochorial (Hill, 1932). Even though pre-eclampsia appears to be a uniquely human disease (Martin, 2003), there are findings where Old World monkeys (Palmer *et al.*, 1979) and great apes (Stout and Lemmon, 1969) show symptoms that resemble pre-eclampsia. However, trophoblast invasion in non-human primates is more restricted than in the humans, and the absence of interstitial trophoblast cells in the monkey is a major difference in comparison to human placentation (Carter, 2007). Ethical issues and the high maintenance costs of primate colonies, as well as concerns for vulnerable or endangered species, limits the use of non-human primates as animal models (Caldecott and Miles, 2005).

#### 1.2.2.3: *In vitro* models of trophoblast differentiation

##### 1.2.2.3.1: EVT's propagated from human first trimester placenta tissues

Irving *et al.* (1995) reported a pure yield of EVT cultures by isolation (cutting the villi with a surgical scissor and mince finely with a razor blade) from first trimester placenta tissues (6-12 weeks gestation), as confirmed through morphological and phenotypical analysis. Indirect immunofluorescence showed that pure trophoblast outgrowths stain

100% positive for the epithelial cell markers cytokeratin 8 and 18, but do not stain for vimentin, a cell marker for mesenchymal cells (Irving *et al.*, 1995). In addition, equal or more than 90% of mechanically isolated EVT<sub>s</sub> from placenta tissue immunostained positive for cytokeratin and insulin-like growth factor-II (Aplin *et al.*, 1999). Therefore, the expression of these markers in isolated EVT<sub>s</sub> from placenta tissues allows the confirmation of a valid model for investigating EVT cell biology.

#### 1.2.2.3.2: Villous cytotrophoblasts isolated from human term placentae

Villous cytotrophoblasts are isolated by a digestive enzyme from human term placental tissues followed by purifying the cells by using either immunoselection or density gradients. These cell isolating methods result in highly purified populations of mononucleate cytotrophoblasts (Kliman *et al.*, 1987; Yui *et al.*, 1994; Morrish *et al.*, 1997). The cellular biology of mononucleate cytotrophoblasts mimics many of the cellular events associated with chorionic villous formation *in vivo*, for example, mononucleate cytotrophoblasts freshly isolated from human term placenta undergo aggregation, differentiation, and fusion to form a multinucleated syncytial trophoblast over time in culture. The level of  $\beta$  human chorionic gonadotropin increases during the formation of multinucleated syncytium in these primary cell cultures (Hoshina *et al.*, 1982).

### 1.2.2.3.3: Human trophoblastic cell lines

#### 1.2.2.3.3.1: Choriocarcinoma cell lines

Progress in understanding of human trophoblast differentiation has been restricted due to the cellular and morphological differences that exist between human placenta and animal models, as well as the fact that *in vivo* human experimentation is difficult to justify. Trophoblastic cell lines derived from choriocarcinoma cells have provided a useful alternative for investigating human trophoblast differentiation *in vitro* (King *et al.*, 2000). Choriocarcinoma is a relatively uncommon malignant tumour of the human placenta that consists of mitotically active cytotrophoblasts (Benirschke and Kaufmann, 2000). The choriocarcinoma cell lines, known as BeWo, JEG-3 and JAR are the most commonly used cell lines.

BeWo choriocarcinoma cells undergo cellular differentiation in response to forskolin or cAMP (cyclic adenosine monophosphate) treatment (Seamon *et al.*, 1981; Wice *et al.*, 1990). In response to forskolin or 8-bromo-cAMP, BeWo cells show a marked reduction in DNA synthesis and within 48-96 hours of treatment these cells begin to fuse and form large syncytia (Coutifaris *et al.*, 1991). This constitutes an *in vitro* model to study the cellular and molecular processes involved in syncytial trophoblast formation.

JEG-3 choriocarcinoma cells are mononucleate trophoblastic cells that were established by Kohler *et al.* (1971). When JEG-3 cells were treated with 8-bromo-cAMP, there was an increase in  $\beta$  human chorionic gonadotropin production; however these cells do not undergo differentiation to form a multinucleated syncytium under these culture

conditions (Chou *et al.*, 1978; Burnside *et al.*, 1985; Coutifaris *et al.*, 1991). Instead, JEG-3 cells have been used as an *in vitro* model to study the cell biology of mononucleate trophoblasts.

JAR is a choriocarcinoma cell line originating from a human malignant cytotrophoblastic tumor. JAR cells have been used, for example, to study the molecular mechanisms involved in iodide transport from mother to fetus (Arturi *et al.*, 2002). In preliminary gain-of-function studies using JAR cells transfected with an expression vector containing full-length Twist cDNA, I found significantly increased Twist mRNA expression, however the TWIST protein level remained unchanged (data not shown) and so these choriocarcinoma cells proved to be an unsuitable experimental system in which to study Twist-dependent effects.

#### 1.2.2.3.3.2: HTR-8 cells

Studies of the trophoblast biology have been traditionally dependent on the use of primary trophoblast cultures from first trimester tissues (Kliman *et al.*, 1986; Yagel *et al.*, 1989). The drawback of using these primary cell cultures is their short life span, as the cells senesce after 5-6 passages. Furthermore, short-term cultures are not appropriate for certain experiments, such as those involving genetic manipulations, because these studies usually require long-term culture. Experiments using primary trophoblast cultures are also accompanied by high variability between samples due to heterogeneity in the cell population. HTR-8 cells, an EVT cell line, also known as parental HTR-8 cells or sometimes referred to as HTR-8/SVneo, when the parental HTR-8 cells are transfected



with Simian Virus 40 (SV 40) large T antigen (Tag). These transfected HTR-8/SVneo cells can normally be maintained in culture for more than 50 passages in comparison to their parental HTR-8 cells which senesced after 12-14 passages. Furthermore, these transfected cells retain most phenotypic features as compared to their non-transfected parental HTR-8 cells (Graham *et al.*, 1993). HTR-8/SVneo cells also display a premalignant phenotype as indicated by hyperproliferative and hyperinvasive behaviour, and resistance to the anti-proliferation and anti-invasive effects of transforming growth factor  $\beta$  (TGF  $\beta$ ) (Khoo *et al.*, 1998). HTR-8/SVneo cells cultured in hypoxic conditions (2% oxygen concentration) exhibit reduced cell invasive properties. These cells also express cytokeratin and retain several important characteristics typical of primary cultures of first-trimester human cytotrophoblast cells, including altering their behaviour in response to a changing maternal environment (Kilburn *et al.*, 2000).

### **1.3: Cellular and molecular mechanisms involved in terminal differentiation of human trophoblasts**

#### 1.3.1: Extracellular matrix degradation

In general, the extracellular matrix (ECM) that surrounds cells is comprised of proteins such as collagen, fibronectin, laminin and proteoglycans. Degradation of the ECM is required during biological processes such as tumour invasion and early placental development. Several key proteinases, such as those of the plasminogen activator (PA) family and the matrix metalloproteinase (MMP) family are activated to promote ECM degradation during these biological processes (MacDonald *et al.*, 1998; Whiteside *et al.*,

2001, Denys *et al.*, 2004). The roles of PA and MMP family members in human trophoblast differentiation are described in the following sections.

#### 1.3.1.1: Plasminogen activators and their inhibitors

The plasminogen activators (PA) are substrate-specific proteinases that mediate cleavage of plasminogen to plasmin (Alfano *et al.*, 2005). PA exhibits a wide range of serine protease activities that target extracellular matrix components such as laminin and fibrin, as well as assisting in the activation of zymogen forms of MMPs (Vaseelli *et al.*, 1991; Andreasen *et al.*, 2000; Durand *et al.*, 2004). The PA system consists of the urokinase-type PA (uPA), the tissue-type PA (tPA), the PA inhibitors-1 and -2 (PAI-1 and PAI-2), and the uPA receptor (uPAR).

uPA is spatiotemporally expressed at the maternal-fetal interface during first trimester pregnancy in humans and higher primates (Hu *et al.*, 1999; Feng *et al.*, 2001). uPA has also been identified in subpopulations of extravillous cytotrophoblasts (EVTs) that invade into decidual tissue and spiral arteries to ensure a continuous supply of blood to the placenta (Fisher and Damsky, 1993). Neutralizing antibodies specific for uPA inhibit the invasive capacity of EVT (Graham *et al.*, 1994). Furthermore, IL-1 $\beta$ , which promotes trophoblast invasion, up-regulates uPA expression in EVT (Karmakar and Das, 2002). This suggests that uPA may play a key role in regulating EVT invasion during pregnancy.

PAI inhibits the proteolytic activity of uPA by forming a complex with uPAR in a covalent manner that results in a conformational change (Andreasen *et al.*, 1990). TGF-

$\beta$ 1, a growth factor that inhibits trophoblast invasion, can up-regulate PAI-1 and PAI-2 in EVT<sub>s</sub> (Graham, 1997; Karmakar and Das, 2002).

In addition, PAI-1 and PAI-2 are differentially expressed during the terminal differentiation and fusion of villous cytotrophoblasts isolated from human term placenta. PAI-1 is highly expressed in freshly isolated mononucleate cytotrophoblasts, while PAI-2 is highly expressed in the terminally differentiated syncytial trophoblast (Feinberg *et al.*, 1989).

#### 1.3.1.2: Matrix metalloproteinases and their inhibitors

Matrix metalloproteinases (MMPs) are a homologous family of proteolytic enzymes. Based on their substrate specificity and structure, over 20 members of the MMP gene family are classified into four subgroups: the collagenases (MMP-1, MMP-8 and MMP-13) that digest type I, II, III, VII, and X collagens, which are major constituents of interstitial ECM; the gelatinases (MMP-2 and MMP-9) that digest type IV collagen, a basement membrane protein; membrane-type MMPs (MMP-14, MMP-15 and MMP-16) that are most commonly assigned a role in activating proMMP-2 by cleaving it at the cell surface; and the stromelysins (MMP-3, MMP-7, MMP-10, MMP-11, and MMP-12) that digest type IV, V and VII collagens, proteoglycans, laminin, fibronectin and elastin (Wang *et al.*, 2000, Bischof *et al.*, 2001; Cohen *et al.*, 2006; Fingleton, 2006). These proteinases hold a zinc atom in a highly conserved active site and are responsible for ECM remodelling (Brown and Giavazzi, 1995). MMPs were initially thought to function mainly as enzymes that degrade the structural components of the ECM. However, these

proteinases have been found to regulate tissue architecture through their effects on the ECM and intercellular junctions, by producing substrate-cleavage fragments, creating spaces for cells to migrate, thereby activating or deactivating signalling molecules, either directly or indirectly (Sternlicht and Werb, 2001).

MMPs are tightly controlled, due to their degradative potential, and are secreted as latent proenzymes. The removal of an amino-terminal domain is required for the enzyme to be activated (Springman *et al.*, 1990; Kleiner and Stetler-Stevenson, 1993). This tight regulation of enzyme activity is necessary in normal physiological situations, such as in wound healing or morphogenesis (Brenner *et al.*, 1989; Bullen *et al.*, 1995). However, there is excessive MMP expression in cancer progression (Brown *et al.*, 1993; Davies *et al.*, 1993). This enables the tumour cells to grow and then invade into the blood circulation and lymphatic system, thus leading to tumour spread.

Current studies show that the relationship between MMP expression and cancer is complex. For example, increased MMP activity may promote or inhibit tumour progression (Coussens *et al.*, 2002) depending on factors such as the tumour site (primary or metastasis), the tumour stage and the MMP substrate profile and enzyme localization (tumor *vs.* stromal) (Fridman, 2006). MMP-2 and MMP-9 have been linked with the processes of cancer cell invasion and metastasis in humans, as these two proteinases have been associated in the progression of cervical uterine cancer (Libra *et al.*, 2009). Furthermore, as the ECM may be the primary barrier to tumour growth and spread, MMPs may assist malignant tumour cells to overcome this barrier, and thus represent therapeutic targets in the treatment of cancer metastasis (Brown and Giavazzi, 1995).

The membrane-type MMPs (MT-MMPs), form a distinct membrane-type subclass in the MMP family since all the others members are secreted in the soluble forms. Instead, MT-MMPs induce the activation of pro-gelatinase A (68-kDa in gelatine zymography) on the cell surface into the activated form of 62-kDa fragments through a 64-kDa intermediate form (Sato *et al.*, 1994; Takino *et al.*, 1995). MT-MMPs have a major impact on cancer development due to their cellular localization at the tumour-matrix interface, for example, MT1-MMP promotes tumour invasion (Fridman, 2006). Furthermore, loss-of-function studies targeting MT1-MMP or MT2-MMP completely abolish the ability of SNAIL to induce carcinoma cell invasion through the underlying basement membrane (Ota *et al.*, 2009).

Several studies have shown that MMP-2 and MMP-9 synthesis and activation are also necessary for trophoblast invasion (Librach *et al.*, 1991, Shimonovitz *et al.*, 1994; Bishop and Campana, 2000; Isaka *et al.*, 2003). These two proteinases are differentially expressed in the first trimester (6-8 weeks) when trophoblast cells with MMP-2 being a key enzyme (gelatinase) are involved in trophoblast invasion through ECM degradation. In the late first trimester (9-12 weeks) trophoblasts, both of these gelatinases, MMP-2 and MMP-9 are involved in trophoblast invasion through degradation of collagen IV, the main component of the basement membrane (Librach *et al.*, 1991; Xu *et al.*, 2000; Staun-Ram *et al.*, 2004). Besides regulating extravillous cytotrophoblast invasion, MMP-2 and MMP-9 are produced by the syncytial trophoblast and have a role in degradation and reformation of the placental basal lamina of chorionic villi (Sawicki *et al.*, 2000).

The MMPs are inhibited by proteinase inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). The TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) share

sequence identity with one another, and have similar activities towards the various members of the MMP gene family. However some specificity is evidenced by the finding that TIMP-1 preferentially binds to MMP-9, while TIMP-2 and TIMP-3, but not TIMP-1, are effective inhibitors of Membrane Type-MMPs (Strongin *et al.*, 1995). Several MMPs and TIMPs are co-expressed in trophoblasts, suggesting that the invasive capacity of cytotrophoblastic cells could depend on the relative expression of various MMPs and TIMPs (Freitas *et al.*, 1999; Terrade *et al.*, 2002).

### 1.3.2: Extracellular matrix deposition

The ECM plays crucial roles in maintaining tissue integrity as well as modulating cellular differentiation during development (Lin and Bissell, 1991; Adams and Watt, 1993). The ECM also modulates the terminal differentiation and fusion of villous cytotrophoblasts isolated from term placenta (Kao *et al.*, 1988). In humans, mononucleate cytotrophoblasts are separated from villous mesenchymal tissue by a basement membrane. Several ECM components including collagen type IV, laminin, heparan sulphate and proteoglycan are found within the basement membrane (Earl *et al.*, 1990; Damsky *et al.*, 1992; Onodera *et al.*, 1997). The glycoprotein fibronectin is, however, expressed at different gestational periods in the basement membrane (Yamada *et al.*, 1987; Virtanen *et al.*, 1988; Earl *et al.*, 1990), and addition of fibronectin to primary cultures of cytotrophoblasts promotes syncytial trophoblast formation *in vitro* (Kao *et al.*, 1988). In addition, oncofetal fibronectin, an alternatively spliced variant of fibronectin, is synthesized and secreted by trophoblast cells in culture, and has been

identified at the contact sites between cytotrophoblast columns and the maternal decidua (Matsuura and Hakomori, 1985; Matsuura et al., 1989; Feinberg et al., 1991), suggesting that oncofetal fibronectin maintains placental-uterine interactions during pregnancy.

Another ECM component known as tenascin has anti-adhesive properties *in vitro* (Aufderheide and Ekblom, 1988). This glycoprotein is expressed in areas beneath degenerating syncytium, at locations where cytotrophoblast cells proliferate (Castelluci *et al.*, 1991; Damsky *et al.*, 1992). This suggests that tenascin may play a direct or indirect role in modulating villous cytotrophoblast differentiation.

### 1.3.3: ECM interactions: the integrin gene superfamily of cell adhesion molecules

One of the best characterized groups of ECM receptors that mediate cell-ECM interactions are the integrins (Lochter, 1999). Integrins are glycoproteins and members of a protein family that forms heterodimeric subunits that interact with various ECM components (van der Flier and Sonnenberg, 2001). In mammals, 16 different integrin  $\alpha$  and 8 different integrin  $\beta$  subunits are currently known (Giancotti, 1997). The  $\alpha$  and  $\beta$  subunits interact with each other noncovalently. Each integrin has a specific set of extracellular ligands, and the ligand specificity of the different integrin heterodimers is determined by the specific combination of  $\alpha$  and  $\beta$  subunits expressed on the cell surface (Lafrenie and Yamada, 1996). Each subunit has a large extracellular domain, a single transmembrane domain, and a short, noncatalytic cytoplasmic tail, apart from the  $\beta 4$  subunit that has a very large cytoplasmic domain (Colombatti *et al.*, 1993). Integrins are involved not only in adhesive functions between the cell and the ECM that provide

traction for movement and cell migration, but they can mediate cell-cell adhesion, and activate signal transduction pathways for anchorage-dependent survival and growth (Rosen *et al.*, 1989).

Integrins are expressed in endometrial, decidual, and extravillous cytotrophoblasts (EVTs). During early pregnancy, different members of integrin subtypes ( $\alpha V\beta 3$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$ ) are expressed in trophoblast-endometrium interfaces. During the differentiation of cytotrophoblasts along the extravillous pathway, expression of various integrins differs between proliferative and endovascular EVT<sub>s</sub> (Merviel *et al.*, 2001). Function-perturbing antibodies to the  $\alpha 5\beta 1$  integrin subtype interrupt the organization of extravillous cytotrophoblast columns that form in chorionic villous explant cultures (Aplin *et al.*, 1999). Aberrant expression of  $\alpha V\beta 3$  is associated with infertility, and women with recurrent miscarriages have a lower expression of  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  integrins in the stroma during the implantation window (Skrzypczak *et al.*, 2001). Several other health issues such as preeclampsia and intrauterine growth restriction are believed to be caused by placental vascular problems that may be explained by abnormalities in integrin patterns (Damsky *et al.*, 1992; Aplin 1994, Merviel *et al.*, 2001). In female mice, lack of a functional integrin  $\beta 1$  gene results in normal early embryonic development, but there is a failure to implant properly, with subsequent embryonic death (Stephens *et al.*, 1995).

Other factors known to be involved in the implantation process may act through integrins. One example includes insulin-like growth factor-1 (IGF-1) mediated migration of EVT<sub>s</sub> that involves the  $\alpha V\beta 3$  integrin pathway (Kabir-Salmani *et al.*, 2003 and 2004).



#### 1.3.4: Cell-cell interactions

Many studies have focused on the expression of various members of the immunoglobulin (*Ig*) gene superfamily of  $\text{Ca}^{2+}$ -independent cell adhesion molecules (CAMs) in human trophoblastic cells (Buck, 1992; Burrows *et al.*, 1996). For example, EVT<sub>s</sub> express vascular (V)-CAM-1, carcinoembryonic antigen (CEA)-CAM, melanoma (Mel)-CAM, platelet-endothelial (PE)-CAM-1, intercellular (I)-CAM, and neural (N)-CAM during the first trimester of pregnancy (Damsky *et al.*, 1992; Burrows *et al.*, 1994; Shih and Kurman, 1996; Coukos *et al.*, 1998; Bamberger *et al.*, 2000). A member of the selectin gene family of  $\text{Ca}^{2+}$ -dependent CAMs called E-selectin, that mediates leukocyte-endothelial cell interactions during inflammation, is also found in these EVT cells (Vestweber, 1992; Varki, 1994; Milstone *et al.*, 2000). There is evidence that E-selectin influences how the blastocyst rolls along the inner uterine wall prior to implantation (Hoozemans *et al.*, 2004). However, the biological function(s) of these CAMs at the maternal-fetal interface remains unclear and requires further investigation.

The regulated expression of gap junction components known as connexins (Cx), that help to establish cell-cell interactions, has been investigated during human trophoblast differentiation and invasion. A Cx subtype known as Cx43 is expressed in isolated human trophoblastic cells (Cronier *et al.*, 1994). However, the role of gap junctions in the terminal differentiation and fusion of human villous cytotrophoblast remains to be elucidated. Although gap junctions between villous cytotrophoblasts and the syncytial trophoblast in the first trimester placenta were detected in early ultrastructural

observations (De Viergilis *et al.*, 1982), these junctions are not present in the trophoblastic cells from human term placenta (Metz *et al.*, 1979 and 1980).

#### **1.4: The cadherin superfamily**

In view of the significant role of cell adhesion molecules involvement in regulating human trophoblast differentiation, we have focused our attention on the gene superfamily of cell adhesion molecules known as the cadherins. Cadherins are a gene superfamily of integral membrane glycoproteins that mediate calcium-dependent cell adhesion through homophilic interactions. The structural components of this gene family include an extracellular domain responsible for cell-cell interactions, a transmembrane domain, and a cytoplasmic domain that is linked to the cytoskeleton (Yagi and Takeichi, 2000). This gene superfamily of cell adhesion molecules consists of two evolutionarily distinct subfamilies: classical cadherins, which are homophilic  $\text{Ca}^{2+}$ -dependent cell-cell adhesion molecules; and non-classical cadherins (Yagi and Takeichi, 2000; Angst *et al.*, 2001). Cadherins are widely known to regulate cell-cell adhesions, regulate cell shape, proliferation, migration, differentiation, and segregation, and are involved in intercellular signalling networks (Takeichi, 1991, 1995).

##### 1.4.1: Classical cadherins

Over 15 members of the classical cadherin subfamily have been identified and are sub-classified into type I and type II cadherins. The most commonly known members of

the cadherin gene superfamily consist of a carboxy terminal cytoplasmic domain, a transmembrane domain, and an amino terminal extracellular domain, (Grunwald, 1993) (Figure 1.2). Some of the classical type I cadherins include E-cadherin (E-cad), N-cadherin (N-cad) and P-cadherin (P-cad). The names of these cell adhesion molecules originated from their tissue distribution during mouse embryonic development; with E-cad mainly expressed in epithelial cells, N-cad in neuronal cells and P-cad in the placenta (Nose and Takeichi 1986, Nagafuchi *et al.*, 1987; Hatta *et al.*, 1988; Suzuki *et al.*, 1991). Type II classical cadherins include cadherin-6, -7, -8, -9, -10, -11, -12, -14, -19, and -20 (Takeichi, 1995).

The classical cadherin subfamily is defined by their highly conserved cytoplasmic domain, which connect with catenins ( $\alpha$ -catenin,  $\beta$ -catenin,  $\gamma$ -catenin [also called plakoglobin], and p120<sup>ctn</sup>) to form the cytoplasmic cell adhesion complex that is necessary for extracellular cell-cell adhesion (Shibamoto *et al.*, 1995; Cavallaro *et al.*, 2002). For example,  $\beta$ -catenin and  $\gamma$ -catenin bind to the same conserved site at the C-terminal region of E-CAD in a mutually exclusive way (Ozawa *et al.*, 1989; Nathke *et al.*, 1994; Witcher *et al.*, 1996), whereas p120<sup>ctn</sup> interacts with multiple sites in the cytoplasmic tail of E-CAD, including the juxtamembrane region (Ozawa, 1998; Yap *et al.*, 1998). It is also known that  $\alpha$ -catenin binds directly to  $\beta$ -catenin and  $\gamma$ -catenin, and thereby connects the cytoplasmic cell adhesion complex to the actin cytoskeleton (Cavallaro *et al.*, 2002).

Studies using X-ray diffraction analysis suggest that the N-CAD extracellular domain forms a dimer, in which two monomers are arranged in parallel at the plasma membrane by forming an “adhesion dimer” at their N-terminus. An alternative model is that the two

monomers are arranged to form a rod- or cylinder-like oligomer rather than two monomers arranged in parallel (Takeichi, 1995). In addition,  $\text{Ca}^{2+}$  is essential for cadherin function by linking the five subdomains to form a rod-shape morphology on the cadherin molecules (Tong *et al.*, 1994).

Type I and type II cadherins consist five extracellular repeats (EC1-5), and the main difference between them is the presence of a histidine, alanine, valine (HAV) tripeptide within the extracellular repeat (EC1) that is closest to the N-terminus. Cadherin molecules with a deletion of EC1 fail to mediate cell-cell interactions, suggesting that the HAV domain interactions between two CAMs may contribute to mediating homophilic protein-protein interaction (Takeichi, 1990; Knudsen *et al.*, 1998). However, the HAV motif is not conserved in Type II cadherins, and the molecular basis of the specificity of cadherin interactions remains unclear. Furthermore, recent X-ray diffraction studies of

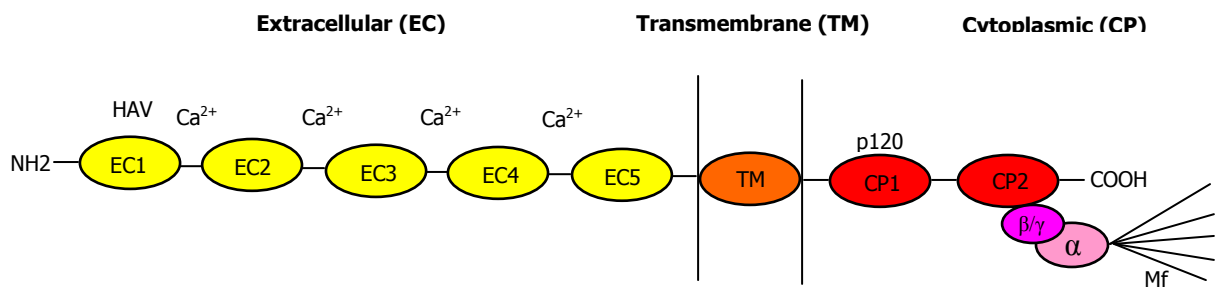


Figure 1.2. Schematic diagram representing the basic structure of type 1 classical cadherins in the plasma membrane. The cadherins are comprised of five extracellular subdomains (EC1-EC5). The EC1 subdomain contains the HAV, which is believed to play a role in cadherin-mediated adhesion. The cytoplasmic subdomains are highly conserved regions interact with a family of cytoplasmic proteins known as the catenins. CP1 interacts with p120<sup>ctn</sup> and CP2 forms complexes with either β or γ-catenin and α-catenin. These interactions are believed to link the cadherins to the actin-based microfilaments (MF) of the cytoskeleton.

the extracellular domain of the *Xenopus* Type I cadherins suggest that other extracellular interfaces are involved in cadherin interactions, including the conserved tryptophan side chain at the membrane-distal end of a cadherin that intercalates into a conserved hydrophobic pocket in the corresponding partner (Boggon *et al.*, 2002).

Classical cadherins undergo calcium-dependent cell-cell adhesion in a homophilic manner, preferentially binding to like molecules, although in some cases a particular cadherin subtype may interact heterophilically with another cadherin subtype when two cell populations are mixed (Takeishi, 1995). Therefore, the interactions between two given cadherins can be classified into three categories: little or no heterophilic interactions; weak heterophilic interactions; and those in which homophilic and heterophilic interactions are not distinguishable (Nakagawa *et al.*, 1995; Takeichi, 1995).

Although the activity of Type I cadherins in tissue formation has been widely studied, with a focus on their cell adhesive properties, this is not the case for many Type II cadherins. However, some of the type II molecules are expressed in loosely associated cells, suggesting a weaker cell-cell interaction compared to type I molecules (Takeichi, 1995; Gumbiner, 1996).

#### 1.4.2: Classical cadherins involvement in developmental processes

In addition to maintaining the structural integrity of cells and tissues, cadherins control a wide range of cellular behaviours (Huber *et al.*, 1996; Larue *et al.*, 1996). They regulate cell polarization (Larue *et al.*, 1994; Riethmacher *et al.*, 1995), and cell

movements including cell sorting and cell migration (Nose *et al.*, 1988; Takeichi, 1988; Steinberg and Takeichi, 1994).

E-cad-deficient mouse embryos cannot normally develop into blastocysts, thus confirming the importance of E-cad in the organization of pre-implantation embryos (Larue *et al.*, 1994; Riethmacher *et al.*, 1995). N-cad, like E-cad, is critical during embryonic development. Indeed, the loss of either of these cadherin subtypes results in early embryonic death (Larue *et al.*, 1994; Radice *et al.*, 1997a). In addition, in mice without functional N-CAD, the myocardium is malformed, causing arrest of heart development, and the neural tube and somites are also not properly formed (Radice *et al.*, 1997a). However, null mutant mice R-cadherin (R-cad) or P-cadherin (P-cad) are viable and fertile (Radice *et al.*, 1997b; Dahl *et al.*, 2002).

#### 1.4.3: Classical cadherins and cancer

The majority of human cancers originate from epithelial cells. These epithelial cells are organized by a number of specific intercellular junctions, including adherens-type-junctions, tight junctions and desmosomes, which are interconnected with the actin and intermediate filament cytoskeleton (Cavallaro *et al.*, 2002). Among the cell-cell adhesion molecules, cadherins appear to play a crucial role in establishing adherens-type-junctions (Takeichi, 1995; Huber *et al.*, 1996; Yagi and Takeichi, 2000). It is well known that cell-cell adhesion is changed markedly during the development of malignant cancers (Cavallaro *et al.*, 2002). For instance, loss of E-CAD-mediated cell-cell adhesion is a requirement for tumour cell invasion and metastasis formation (Birchmeier and Behrens,

1994). Recent evidence demonstrates that N-cad is dominant over E-cad in metastatic progression and is overexpressed in a subset of cancer types in addition to the loss of E-cad (Derycke *et al.*, 2004; Hazan *et al.*, 2004). Nieman *et al.* (1999) reported that forced expression of N-cad causes E-cad to be down-regulated in breast cancer cells. The increase of N-cad expression increases the resistance to apoptotic stimuli, and a more invasive and motile cell phenotype and metastasis in nude mice (Cavallaro *et al.*, 2002 and 2004; Jiang *et al.*, 2007), thus demonstrating opposite effects to E-cad. It has been suggested in that a switch from epithelial to mesenchymal cadherins supports the transition from benign to an invasive, malignant tumor phenotype.

In contrast, Rosivatz *et al.* (2004) suggest that E-CAD transcriptional repression may not play a major role in colon cancer pathogenesis, and other studies did not always observe a correlation between reduced E-CAD immunohistochemistry and tumor progression. However, in the latter study, the same tumors had N-CAD immunoreactivity in 44% of the cases. It seems that N-cad has an invasion promoting effect, as earlier shown for other carcinomas, and that N-cad induced invasion activities can even overcome the E-CAD tumor suppressive function (Hazan *et al.*, 1997; Nieman *et al.*, 1999). This suggests that the “cadherin switch” can vary in a tumor-specific manner.

As discussed previously, villous cytotrophoblasts differentiating along the EVT pathway utilize similar molecular mechanisms to those employed in cancer cells (Bischof *et al.*, 2001; Lala *et al.*, 2002). Unlike cancer cell invasion, normal EVT invasion into the maternal endometrium and vasculature is highly regulated (Graham *et al.*, 1993; Irving *et*



*al.*, 1995). We therefore speculate that cadherins play an important role in human trophoblast invasion.

Several mechanisms are involved in the loss of E-CAD function during tumorigenesis; these include mutation or deletions of the E-cad gene itself, transcription repression of the E-cad gene, hypermethylation of CpG islands or chromatin rearrangements in the E-cad promoter region, as well as in the  $\beta$ -catenin gene (Hirohashi, 1998).

Several studies have highlighted transcription repression as the major mechanism leading to decreased E-cad expression (Schipper *et al.*, 1991; Bussemakers *et al.*, 1992; Brabant *et al.*, 1993). E-cad transcription repressors belong to three families: i) TWIST; ii) SNAIL (SNAIL1, SNAIL2 (SLUG), SNAIL3), and ZEB1 (DeltaEF1)/ ZEB2 (SIP1), and interact with the E-cad gene promoter (Cano *et al.*, 2000; Comjin *et al.*, 2001; Bolos *et al.*, 2003). For example, in many human carcinomas of the ovary, liver, colon, and gastrointestinal tract, Snail expression correlates with inhibition of E-cad expression (Blanco *et al.*, 2002; Jiao *et al.*, 2002). Furthermore, TWIST has been shown to transcriptionally repress E-cad in breast cancers (Yang *et al.*, 2004; Vesuna *et al.*, 2008).

In human gastric cancer, an increase in Snail mRNA expression is associated with a down-regulation of E-cad. An increase in N-cad mRNA levels was also detected in the same tumours, likely due to the overexpression of Twist (Rosivatz *et al.*, 2002). Twist also induces N-cad expression in prostate carcinoma cells (Alexander *et al.*, 2006). This suggests these EMT regulators could play different roles in gastric carcinogenesis depending on the histological subtype (Rosivatz *et al.*, 2002). In relation to the mechanism that regulates E-cad, *in vivo* footprinting analysis shows that the positive

regulatory elements of the E-cad promoter (the CCAAT-box) were bound by transcription factors in cells that expressed E-cad but not in non-expressing cells (Hirohashi, 1998).

#### 1.4.4: Classical cadherins and placentation

It is acknowledged that morphogenesis and cell differentiation depend, in part, on the regulated expression of cell surface glycoproteins and their connections to the cytoskeleton (Edelman, 1988). Some of these adhesion molecules are well-characterized including E-CAD (Damsky *et al.*, 1984; Takeichi, 1991; Kemler *et al.*, 1989).

In regard to embryonic development and placental morphogenesis, E-CAD has been gaining much attention (Coutifaris *et al.*, 1991) because antibodies against E-CAD have shown to inhibit the compaction of preimplantation mouse embryos: a developmental process that involves blastomere adhesion and formation of intercellular junctions (Hyafil *et al.*, 1980; Damsky *et al.*, 1983; Vestweber and Kemler, 1984). In addition, E-cad mRNA and protein levels are high in freshly isolated villous cytotrophoblasts and decrease as the cells begin to undergo differentiation and fusion to form multinucleated syncytia (Coutifaris *et al.*, 1991; Rebut-Bonneton *et al.*, 1993). Similarly, E-CAD has been localized on the surface of cytotrophoblasts *in situ*, but not on the surface of the syncytiotrophoblast (Edelman *et al.*, 1989). Furthermore, immunoneutralization experiments using an antiserum directed against the cell adhesion domain of cadherins found inhibition of the formation of syncytia in comparison to an antiserum against the cytoplasmic tail of E-cad, which had no effect upon aggregation and fusion of these cells

(Blaschuk *et al.*, 1990; Pouliot *et al.*, 1990; Coutifaris *et al.*, 1991; Farookhi and Blaschuk, 1991).

A type II classical cadherin known as cadherin-11 (Cad-11) plays an important role in mediating trophoblast-endometrium interactions. Its expression increases during the formation of multinucleated syncytia in primary cultures of human cytotrophoblasts. When cad-11 was transfected into poorly invasive JEG-3 choriocarcinoma cells, this resulted in the formation of multinucleated syncytia in the transfected JEG-3 cell cultures (MacCalman *et al.*, 1996; Getsios and MacCalman, 2003). This suggests that cad-11 contributes to the morphological and functional differentiation of the multinucleated syncytial trophoblast.

### **1.5: TWIST- A basic helix-loop-helix transcription factor**

The Twist gene encodes a transcription factor that was originally identified in *Drosophila*. The human Twist gene is located at 7p21 and comprises two exons that are separated by an intron. TWIST contains a conserved basic helix-loop-helix (bHLH) domain (Bourgeois *et al.*, 1996) (Figure 1.3). The function of bHLH transcription factors depends on the DNA-binding region and an HLH motif that mediates homodimerization or heterodimerization with other HLH proteins to form a functional dimer that can recognize and bind to a DNA motif called the E-box (Benezra *et al.*, 1990; Massari and Murre, 2000; Castanon and Baylies, 2002).

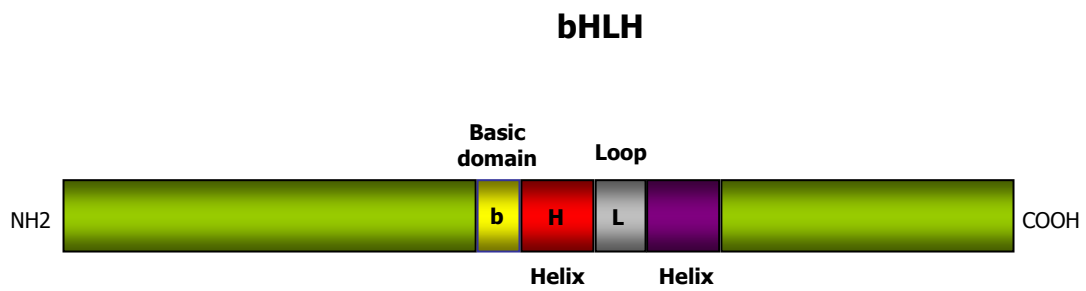


Figure 1.3. Schematic diagram representing of the protein structure of the human Twist. The TWIST protein has 202 amino acids with a basic helix-loop-helix domain. The basic (b) is the DNA-binding region and the helix-loop-helix mediates homodimerization or heterodimerization with other HLH proteins to form a functional dimer.

Post-translational modifications like phosphorylation can alter the dimerization choices of TWIST, either promoting homodimer or heterodimer formation (Firulli *et al.*, 2005), and different TWIST dimerization partners could have specific effects on the transcription regulatory function of TWIST. It has been suggested that TWIST as a heterodimer functions as a transcription repressor, while TWIST homodimers favour the upregulation gene expression by functioning as a transcription activator. For example, in studies of human cranial suture patterning and *Drosophila* mesoderm development, TWIST homodimers function as transcriptional activators (Castanon *et al.*, 2001; Connerney *et al.*, 2006).

#### 1.5.1: Twist- A key player in cell differentiation and morphogenesis

Twist is expressed in mesodermal and cranial neural crest cells during embryogenesis in vertebrate and invertebrate development (Thisse *et al.*, 1988; Fuchtbauer, 1995). Studies have shown that mutated Twist results in a twisted phenotype in *Drosophila* embryos, suggesting the expression pattern of Twist is associated with the formation and specification of the mesoderm (Thisse *et al.*, 1988; Leptin, 1991). Heterozygous Twist mutant mice show a number of craniofacial defects, including a narrow palate and craniosynostosis. This is similar to patients with a skeletal dysplasia termed Saethre-Chotzen's syndrome, in which reported mutations were found to involve the bHLH domain of the Twist gene (El Ghouzzi *et al.*, 1997; Bourgeois *et al.*, 1998).

Furthermore, Bialek *et al.* (2004) demonstrated that the molecular defect in the Saethre-Chotzen syndrome is caused by haploinsufficiency at the Twist locus. This gene

mutation which leaves TWIST's bHLH domain intact but disrupts its TWIST box results in a serious form of the disease, with limb patterning defects (Gripp *et al.*, 2000; Bialek *et al.*, 2004). Bialek *et al.* (2004) has also shown that TWIST inhibits osteoblast differentiation by interfering with RUNX2, a member of the RUNX family of transcription factors which function through interacting with specific domains in these proteins; i.e., the TWIST box (a domain distinct from their bHLH domains) and a RUNX2 DNA binding domain.

### 1.5.2: Twist and cancers

Twist is linked to metastases of a wide range tumour types including those of ovary, breast and prostate tissues (Kwok *et al.*, 2005; Puisieux *et al.*, 2006; Hosono *et al.*, 2007). Recently, Twist was shown to play a key role in inducing cell movement and tissue reorganization during invasion and metastasis in breast cancer. Indeed Twist clearly appears to be one of the most strongly up-regulated genes responsible for invasiveness and/or intravasation of mouse mammary tumors (Yang *et al.*, 2004). Twist also has anti-apoptotic effects and can play a role in cell survival (Maestro *et al.*, 1999). Yang *et al.* (2004) reported that suppression of Twist expression in highly metastatic mammary carcinoma cells specifically inhibits their metastasis to the lung. Furthermore, inhibition of Twist expression using small interfering RNA (siRNA) significantly impairs the metastatic ability of the most metastatic tumour cell lines (Yang *et al.*, 2004).

EMT is characterized by the gain of mesenchymal cell markers such as N-CAD, vimentin, smooth muscle actin, and fibronectin; and the loss of epithelial markers such as

E-CAD and catenins (Thiery, 2003; Ridisky, 2005). The loss of E-CAD protein and/or transcriptional repression of E-cad mRNA are hallmarks of EMT, both in cancer progression and embryonic development (Thiery, 2002 and 2003). As mentioned previously, a major mechanism leading to decreased E-CAD levels seems to be a decrease in E-cad transcription (Bussemakers *et al.*, 1993). TWIST is known to play a key role in E-cad repression and EMT induction (Yang *et al.*, 2004). Inactivation of Twist reduces migration and invasion abilities of androgen-independent prostate cancer cells, and is correlated with induction of E-cad expression as well as morphologic and molecular changes linked with EMT (Kwok *et al.*, 2005). In line with its function in EMT, TWIST represses transcription from the E-cad promoter *via* the E-boxes that are also targeted by SNAIL and SIP1 (Comijn *et al.*, 2001). In addition, high TWIST levels are also linked with deep myometrial invasion of cancer cells and were concurrent with decreased E-CAD levels, a hallmark of EMT (Satoru *et al.*, 2006).

The gain of N-cad expression, a mesenchymal marker, may increase motility and invasion of carcinoma cells as well (Cavallaro *et al.*, 2002). The switch from E-CAD to N-CAD is mediated by a number of transcription factors, including TWIST, SIP1 and SNAIL1 (Rosivatz *et al.*, 2004). As mentioned earlier, TWIST has been shown to be essential for the initiation of N-cad expression in *Drosophilla* (Oda *et al.*, 1998) and in cancer cell invasion (Alexander *et al.*, 2002; Rosivatz *et al.*, 2002). The effect of TWIST on inducing N-cad is exerted by a direct interaction with an E-box *cis*-element located within the first intron of the N-cad gene (Alexandra *et al.*, 2006).

Since Twist has regulatory and functional roles in both cancer differentiation and normal differentiation, we speculate that Twist may play an important role in human trophoblast differentiation.

### **1.6: Runt-related Gene (Runx) family**

The Runx family is composed of three closely related genes; Runt-related gene 1 (Runx1), Runt-related gene 2 (Runx2), and Runt-related gene 3 (Runx3), each with tissue-specific functions and are homologous to the *Drosophila* gene runt. RUNX proteins are scaffolding transcription factors, which contain a runt homology domain that serves as the DNA-binding domain. They localize to subnuclear domains and translate cell signals through the formation of gene promoter regulatory complexes (Zaidi *et al.*, 2001 and 2003). An essential feature of RUNX proteins is their C-terminal nuclear matrix targeting signal (NMTS) domain that mediates the organization of regulatory complexes (Zaidi *et al.*, 2004) (Figure 1.4). They can form heterodimers with the transcriptional co-activator core binding factor  $\beta$  (CBF  $\beta$ ) / polyoma enhancer binding protein 2 $\beta$  (PEBP 2 $\beta$ ) *in vitro* (Komori, 2006).

RUNX proteins control different aspects of embryonic development, and they are usually anti-proliferative in cellular differentiation in normal tissues. In addition, these transcription factors are known for their pathogenic function in different human diseases (Guo *et al.*, 2002; Pratap *et al.*, 2003; Wotton *et al.*, 2004). RUNX proteins are known to be involved in haematopoiesis (RUNX1), osteogenesis (RUNX2), and neurogenesis (RUNX3), and are also involved in other developmental processes. For example,



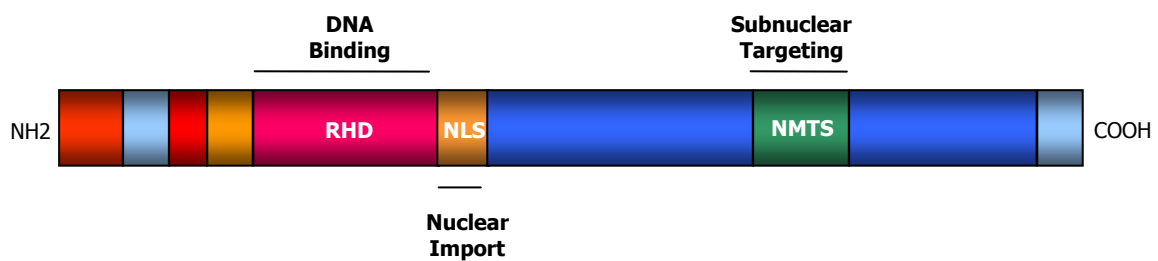


Figure 1.4. Schematic diagram representing of the protein structure of the human Runx2. The RUNX2 protein consists of runt homology domain (RHD) that serves as the DNA-binding domain, the nuclear localization signal (NLS) that directs newly synthesized protein into the nucleus and the nuclear matrix targeting signal (NMTS) domain that mediates the organization of regulatory complexes.

conditional expression of Runx1 in an endothelial progenitor cell line from the aorta-gonad mesonephros region in mice, initiated Vascular Endothelial-cadherin expression and greatly enhanced the vascular network formation activity of the cells, suggesting that Runx1 plays a role in angiogenesis through Vascular Endothelial-cadherin (Iwatsuki *et al.*, 2005). Furthermore, RUNX1 and RUNX3 have both been identified to play a role in the nervous system of mice. RUNX1 plays a role in the nociceptive development of the sensory neurons, however, RUNX3 is responsible for the proprioceptive sensory neurons. Both of these developmental processes are critical in neuronal development (Inoue and Ito, 2008).

RUNX proteins can function as cell context-dependent tumour suppressors or oncogenes (Blyth *et al.*, 2005). Aberrant expression of Runx genes is associated with cell transformation and tumour progression (Pratap *et al.*, 2006). For example, Runx1 mutations and chromosomal translocations have been associated with leukemia subtypes. Amplification of Runx1 is associated with poor prognosis in childhood B-cell leukemia (Osato and Ito, 2005), and loss of Runx3 predisposes mice to gastric hyperplasia (Lau *et al.*, 2006).

Recently, the study of RUNX3 in tumour pathogenesis is gaining more attention in the field of cancer research. The RUNX3 transcription factor has been identified to be a downstream effector of the transforming growth factor- $\beta$  (TGF- $\beta$ ) signalling pathway in controlling cell proliferation, cell invasion, cell adhesion and apoptosis. This transcription factor has also been shown to play a role as a tumour suppressor in colorectal and gastric cancers (Subramanian *et al.*, 2009). However, RUNX3 functional

inactivation caused by mutation or epigenetic silencing has been seen in a wide range of tumour origins from early progression to malignancy (Chuang and Ito, 2010).

#### 1.6.1: Runt-related Gene 2 (Runx2)

Runx2 expression was initially reported in T cells during thymic development and regulates the osteoblast-specific expression of osteocalcin (Ducy and Karsenty, 1995; Satake *et al.*, 1995; Ducy *et al.*, 1997). Loss of function mutations of Runx2 are linked to Cleidocranial dysplasia syndrome in humans (Otto *et al.*, 2002).

Runx2 provides an important mechanistic linkage between cell fate, proliferation, growth control, and lineage commitment. Runx2 has been characterized in bone tissue and is expressed in mesenchymal lineage cells promoting the osteoblast phenotype for bone formation (Otto *et al.*, 1997, Pratap *et al.*, 2006). RUNX2 is the most specific molecular marker of osteoblast lineage; it is needed to induce osteoblast differentiation as well as to regulate most of the genes characteristic of the osteoblast phenotype (Ducy *et al.*, 1997; Komori *et al.*, 1997; Otto *et al.*, 1997). Runx2<sup>-/-</sup> mice demonstrate a complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation (Otto *et al.*, 1997). RUNX2 can also act as a suppressor of ribosomal RNA (rRNA) synthesis (Zaidi *et al.*, 2001; Young *et al.*, 2007). It is also highly expressed in several other tissue types, including the testis (Ogawa *et al.*, 2000), mammary epithelium (Inman and Shore, 2003), endothelial cells (Sun *et al.*, 2001), and prostate and breast tumours (Yeung *et al.*, 2002; Barnes *et al.*, 2003).

Runx2 also promotes vascular endothelial growth factor (VEGF) expression in hypertrophic chondrocytes to mediate angiogenesis during bone synthesis. Runx2 is highly expressed in human and mouse models of angiogenesis, suggesting a possible function for RUNX2 in neovascularisation of adult tissues. Inactivation of the Runx2 gene in mice leads to an absence of vascularisation, in addition to the abnormal bone formation described above (Ducy *et al.*, 1997; Zelzer *et al.*, 2001).

#### 1.6.1.1: Runx2 and cancers

The proper control of gene expression by transcriptional regulators is crucial in balancing the physiological levels of proteins necessary for normal cell function. However, the expression and activities of master transcription factors such as RUNX, TWIST and HOX can become irregular due to mutation, epigenetic changes in chromatin or chromosomal translocations, and can alter the expression of their downstream targets in cancer cells (Yang *et al.*, 2004; Blyth *et al.*, 2005; Grier *et al.*, 2005). The oncogenic potential of Runx2 is suggested by high levels of endogenous RUNX2 in breast and prostate cancer cells associated with aggressive tumour growth in bone (Barnes *et al.*, 2004; Javed *et al.*, 2005). However, tumours do not appear in heterozygotes with haploinsufficiency of Runx2, suggesting Runx2 does not have a role in tumour suppression (Blyth *et al.*, 2005).

In cancer cells, Runx2 can activate expression of adhesion proteins, matrix metalloproteinases and angiogenic factors that are related to the invasive properties of metastatic cancer cells (Pratap *et al.*, 2006a), and MMPs have long been implicated in

tumour invasion and metastasis (Egeblad and Werb, 2002). RUNX2 transcription factor plays a role in regulating MMP-9 expression and promoting cell invasion in bone metastatic cancer cells (Pratap *et al.*, 2005). This is important because one of the processes required for trophoblast invasion is the degradation and remodelling of the MMPs (Cohen *et al.*, 2006).

#### 1.6.1.2: Regulation and activation of Runx2

Increased transcription, epigenetic modifications in chromatin, or silencing of Runx2 repressor proteins have been linked to upregulation of Runx2 expression in breast and prostate cancer cells (Spencer and Davie, 2000; Young *et al.*, 2005). Dysregulation of post-translational modifications (acetylation, ubiquitination, and phosphorylation) can affect the transcriptional activity of Runx2 in cancer cells (Bae and Lee, 2006; Jeon *et al.*, 2006).

TGF- $\beta$  treatment decreases Runx2 expression in osteoblasts, and repeated TGF- $\beta$  treatment also decreases the amount of functional RUNX2 binding to DNA (Komori, 2006). In addition, Runx2 mediates the responses of cells to signal pathways that are often hyperactive in tumours, including those initiated by TGF- $\beta$  and other growth factors. The Smads act as effectors of TGF- $\beta$  to induce changes in gene expression. TGF- $\beta$  binds to the T $\beta$ RI/ T $\beta$ RII receptor complex at the cell surface and activates SMAD 2 and SMAD 3 through phosphorylation, these then form complexes with SMAD 4 and translocate into the nucleus. These complexes link with transcription factors to regulate gene expression through DNA binding (Feng and Derynck, 2005).

### 1.6.1.3: Interactions between RUNX2 and TWIST

The Saethre-Chotzen (SC) syndrome is characterized by increased osteogenesis and premature fusion of cranial sutures caused by Twist mutations (Yousfi *et al.*, 2002). Decreased Twist production causes a narrow sutural space and fusion of bone domains (Yoshida *et al.*, 2004). Other studies have shown that RUNX2 activity is necessary for the regulation of osteoblast differentiation and in the osteogenic switch (Lian *et al.*, 2004). Bialek *et al.* (2004) suggested Twist may regulate the developmental action of RUNX2 in bone formation.

The TWIST box located within the carboxyterminal 20 residues of TWIST is necessary for anti-osteogenic function in mice (Bialek *et al.*, 2004), and it is known to bind with the runt DNA-binding domain of RUNX2 to inhibit RUNX2 transactivation activity (Lian *et al.*, 2004). A decreased level of TWIST may promote RUNX2 function through the lack of inhibitor protein and by the enhancement of its own promoter activity (Yoshida *et al.*, 2005).

A point mutation in the TWIST box leads to an acceleration of bone formation in heterozygous and homozygous mice (Bialek *et al.*, 2004). Furthermore, TWIST box mutations in humans prevent the ability of TWIST to inhibit RUNX2 transactivation (Seto *et al.*, 2007). Cell-free pull-down assays with recombinant RUNX2 and TWIST proteins have suggested a direct physical interaction between RUNX2 and TWIST.

In contrast, Komari *et al.* (2007) show that Runx2 gene expression was unaltered upon transient knock down of Twist in periodontal ligament (PDL) cells using short

interference RNA (siRNA), suggesting that regulatory interactions between TWIST and RUNX2 are tissue or cell dependent.

### 1.7: Hypothesis and rationale:

A successful implantation depends upon mononucleate cytotrophoblasts entering one of two distinct and mutually exclusive pathways. The villous cytotrophoblastic cells will proliferate and differentiate by fusion to form the outer syncytial trophoblast, or enter the extravillous pathway to form highly invasive EVT<sub>s</sub>.

Previous studies have shown that the down-regulation of E-cad is associated with the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro*. There is increasing evidence to suggest that this subpopulation of cytotrophoblasts develop an invasive phenotype *via* molecular and cellular mechanisms adopted by metastatic tumour cells. N-cad has been assigned an integral role in tumour progression and the onset of metastasis but its role in human trophoblastic cell invasion is not known. In view of these observations, the regulated and inverse expression of E-cad and N-cad is a possible mechanism that mediates terminal differentiation of human trophoblastic cells *in vitro*.

The functions of these classical cadherins are known to be regulated, at least in part, by transcription factors in normal development and in cancer progression. The basic helix-loop-helix transcription factor TWIST, as well as the scaffold transcription factor RUNX2, play integral roles in the onset and progression of cancer in a wide variety of tissues. This has prompted the following hypotheses:

1. That the expression of E-cad and N-cad is tightly regulated by TWIST and/or RUNX2 during the terminal differentiation of human trophoblastic cells *in vitro*, which was investigated as described in Chapters 3 and 4 using BeWo cells, primary EVT<sub>s</sub> and HTR-8/SVneo cells.



2. That N-cad plays a key role in regulated development of an invasive phenotype in EVT<sub>s</sub>, which was investigated as described in Chapter 4 using primary EVT<sub>s</sub> and HTR-8/SVneo cells.

3. That Runx2 plays a key role in regulated development of an invasive phenotype in EVT<sub>s</sub>, which was investigated as described in Chapter 5 using primary EVT<sub>s</sub> and HTR-8/SVneo cells.

The specific objectives of my studies were:

1. To identify a role for Twist in regulating the cadherin-mediated terminal differentiation of human trophoblastic cells (in both villous and extravillous pathways).
2. To identify a role for N-cad in regulating human trophoblastic cell invasion *in vitro*.
3. To identify a role for Runx2 in regulating human trophoblastic cell invasion *in vitro*.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1: Tissues**

Samples of first trimester placental tissues were obtained from women undergoing elective termination of pregnancy (gestational ages ranging from 6-12 weeks). The use of these tissues was approved by the committee for Ethical Review of Research on the use of Human Subjects, University of British Columbia, Vancouver, BC, Canada. All women provided informed written consent.

### **2.2: Cells**

Cultures of EVT<sub>s</sub> were propagated from first trimester placental explants as previously described (Getsios *et al*, 1998). Briefly, chorionic villi were washed three times in PBS. The villi were finely minced and plated in 25cm<sup>2</sup> tissue culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, L-glutamine, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and supplemented with 10% fetal bovine serum (FBS). The fragments of the chorionic villi were allowed to adhere for 2-3 days, after which any non-adherent tissue was removed. The villous explants were cultured for a further 10-14 days with the culture medium being replaced every 48 h. The EVT<sub>s</sub> were separated from the villous explants by a brief (2-3 min) trypsin digestion at 37°C and plated in 60 mm<sup>2</sup> culture dishes in DMEM supplemented with antibiotics and 10% FBS. The purity of the EVT cultures was determined by

immunostaining with a monoclonal antibody directed against human cytokeratin filaments 8 and 18 (data not shown). These cellular markers have been used to determine the purity of human EVT cultures (MacCalman *et al.*, 1996). Only cell cultures that exhibited at least 90% immunostaining for cytokeratin were included in these studies.

HTR-8/SVneo, an EVT cell line, was obtained as a gift from Dr Charles H. Graham (Queen's University, Kingston, ON, Canada). Culturing of HTR-8/SVneo has been described previously (Graham *et al.*, 1993). HTR-8/SVneo cells were harvested from ongoing cultures with 0.125% (w/v) trypsin in EDTA buffer. HTR-8/SVneo cells were cultured in 100mm culture dishes (Becton Dickinson and Co, Franklin Lakes, NJ, USA) containing Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose, L-glutamine, antibiotics (100U/ml penicillin, 100 µg/ml streptomycin) and supplemented with 10% fetal bovine serum (FBS).

BeWo and JEG-3 choriocarcinoma cell lines (American Type Culture Collection, Rockville, MD, USA) were harvested from ongoing cultures with 0.125% (w/v) trypsin in EDTA buffer. BeWo and JEG-3 cells were cultured in 100mm culture dishes (Becton Dickinson and Co, Franklin Lakes, NJ, USA) containing F12 Kaighn's medium (F12K) containing L-glutamine, antibiotics (100U/ml penicillin, 100 µg/ml streptomycin) and supplemented with 10% fetal bovine serum (FBS).

### 2.3: Experimental culture conditions

BeWo cells ( $2.5 \times 10^5$  cells) were plated in 35 mm<sup>2</sup> tissue culture dishes and grown to 80% confluency. Cells were treated with or without 8-bromo-cAMP (1.5mM; Sigma-Aldrich, St Louis, MO, USA) for 0, 12, 24, 36 or 48 h. According to the manufacturer, cell-permeable 8-bromo-cAMP has greater resistance to hydrolysis by phosphodiesterases than cAMP. The dosage (1.5mM) of 8-bromo-cAMP was chosen based on previous studies from my laboratory.

EVTs ( $5 \times 10^6$  cells) (passages 4-6) were plated in 60 mm<sup>2</sup> tissue culture dishes (Becton Dickinson and Co, Franklin Lakes, NJ, USA) and grown to 80% confluency. The cells were then washed with PBS and cultured in DMEM under serum-free conditions. Twenty four hours after the removal of serum from the culture medium, the cells were again washed with PBS before cultured in the presence of TGF- $\beta$ 1 (0.001, 0.01, 0.1, 1 or 10 ng/ml) or IL-1 $\beta$  (1, 10, 100 or 1000 IU/ml) for 24 h or TGF- $\beta$ 1 (5 ng/ml) for 0, 6, 12, 24, or 48 h or IL-1 $\beta$  (100 IU/ml) for 0, 12, 24, or 48 h. EVTs cultured in the presence of vehicle (0.1% ethanol) served as controls for these studies. The treatment time and dosage for TGF- $\beta$ 1 and IL-1 $\beta$  were chosen based on previous studies from my laboratory.

To inhibit the regulatory effects of TGF- $\beta$ 1 and IL-1 $\beta$  on *Runx2* mRNA and protein levels in these primary cell cultures, EVTs were cultured in the presence of either TGF- $\beta$ 1 (10 ng/ml) alone or in combination with a function-perturbing monoclonal antibody directed against human TGF- $\beta$ 1 (10  $\mu$ g/ml; Sigma Aldrich) or IL-1 $\beta$  (100 IU/ml) alone or

in combination with a function-perturbing monoclonal antibody directed against human IL-1 $\beta$  (1 or 2  $\mu$ g/ml; Sigma Aldrich) for 24 h.

The time points and the concentrations of cytokines and corresponding function-perturbing antibodies used in these studies were based upon previous reports (Huang *et al*, 1998; Chung *et al*, 2001). All of the EVT cell cultures were harvested for either total RNA or protein extraction.

#### **2.4: Generation of first-strand complementary DNA (cDNA)**

Total RNA was prepared from placenta tissues, cultures of EVTs and choriocarcinoma cell lines using an RNeasy Mini Kit (Qiagen, Inc, CA, USA) following a protocol recommended by the manufacturer. The total RNA extracts were then treated with deoxyribonuclease-1 to eliminate possible contamination with genomic DNA. To verify the integrity of the RNA, aliquots of the total RNA extracts were electrophoresed in a 1% (w/v) denaturing agarose gel, containing 3.7% (v/v) formaldehyde, and the 28 S and 18 S ribosomal RNA subunits visualized by ethidium bromide staining. The purity and concentration of total RNA in each of the extracts were determined by optical densitometry (260/280nm) using a Du-64 UV-spectrophotometer (Beckman Coulter, Mississauga, ON, Canada).

Aliquots (~1  $\mu$ g) of the total RNA extracts prepared from placenta tissues and each of the trophoblastic cell cultures were then reverse-transcribed into cDNA using the First Strand cDNA Synthesis Kit, according to the manufacturer's protocol (Amersham Pharmacia Biotech, Oakville, ON, Canada).

## 2.5: Primer design

Oligonucleotide primers for human Twist, Runx2, E-cad and N-cad were produced according to sequences deposited in GenBank (Accession No.: NM\_000474, NM\_004348, NM\_004360 and NM\_001792 respectively; National Center for Biotechnology Information, Bethesda, MD, USA). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control (Getsios *et al*, 1998b). Oligonucleotide primers corresponding to the nucleotide sequences for Twist, Runx2, E-cad and N-cad were synthesized at the Nucleic Acid and Protein Synthesis Unit, University of British Columbia, Vancouver, Canada. The nucleotide sequences of these primers and the expected sizes of the PCR products are listed in Table 2.1.

A second set of primers specific for Runx2, N-cad or GAPDH, were also prepared in Table 2.2. These primers were used for the real-time quantitative (q) RT-PCR.

Table 2.1. Oligonucleotide primers for Twist, Runx2, E-cad, N-cad and GAPDH mRNA amplification (Semi-quantitative PCR)

mRNA	Primers (5'-3')	Size (bp)	Position on cDNA	No. of cycles
Twist				
Upstream (5 end)	AGTCCGCAGTCTTACGAGGA	576	646-665	30
Downstream (5 end)	GCAGAGGTGTGAGGATGGT		1222-1204	
E-cad				
Upstream (5 end)	TGGATGTGCTGGATGTGAAT	560	1548 -1567	30
Downstream (5 end)	ACCCACCTCTAAGGCCATCT		2107-2088	
N-cad				
Upstream (5 end)	ACAGTGGCCACCTACAAAGG	391	654-673	30
Downstream (5 end)	TGATCCCTCAGGAAGTGTCC		1045-1026	
GAPDH				
Upstream (5 end)	ATGTTTCGTCATGGGTGTGAACCA	378	449-471	22
Downstream (5 end)	TGGCAGGTTTTTCTAGACGGCAG		821-799	

Table 2.2. Real-time qPCR primers for Runx2, N-cad and GAPDH mRNA

mRNA	Primers (5'-3')
Runx2	
Upstream (5 end)	AGCCCTCGGAGAGGTACCA
Downstream (5 end)	TCATCGTTACCCGCCATGA
N-cad	
Upstream (5 end)	TGGGAATCCGACGAATGG
Downstream (5 end)	GCAGATCGGACCGGATACTG
GAPDH	
Upstream (5 end)	GAGTCAACGGATTTGGTCGT
Downstream (5 end)	GACAAGCTTCCC GTTCTCAG

## 2.6: Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using the primer sets specific for Twist, Runx2, E-cad, N-cad or GAPDH, and template cDNA generated from the total RNA extracts prepared from BeWo, JEG-3, EVT<sub>s</sub>, HTR-8/SVneo cell cultures or placenta tissue (villi). The PCR cycles were repeated 15-40 times to determine a linear relationship between the yield of PCR products from representative samples of these cells or tissue and the number of cycles performed. The optimized numbers of cycles subsequently used to amplify Twist, Runx2, N-cad, E-cad and GAPDH are listed in Table 2.1.

All PCR reactions were performed on three separate occasions. PCR was also performed using the primer sets specific for Twist, Runx2, E-cad or N-cad and aliquots of total RNA extracts prepared from BeWo, JEG-3, EVT<sub>s</sub>, HTR-8/SVneo cell cultures or placenta tissue (villi) (i.e. non-transcribed RNA) or DEPC-treated water (negative control) under the same conditions as described above.

An aliquot (10  $\mu$ l) of the Twist, Runx2, E-cad or N-cad PCR products was subjected to electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. The intensity of ethidium bromide staining of the PCR products was analysed by UV densitometry (Biometra, Whiteman Co., Frederick, MD, USA). The absorbance values obtained for Twist, Runx2, E-cad or N-cad were then normalized relative to the corresponding GAPDH absorbance value.



## 2.7: Real-time-quantitative (q) RT-PCR

The first-strand cDNA generated from the HTR-8/SVneo cell cultures served as a template for qRT-PCR using the ABI PRISM 7000 sequence detection system (PerkinElmer Applied Biosystems, Foster City, CA) equipped with a 96-well optical reaction plate. Real-time qPCR was performed using 12.5  $\mu$ l SYBR Green PCR master mix (PerkinElmer Applied Biosystems), 7.5  $\mu$ l of primer mixture (300nM), and 5  $\mu$ l of cDNA template [diluted 1:7 (vol/vol)] under the following optimized conditions: 52 C for 2 min followed by 95 C for 10 min and 40 cycles of 95 C for 15 sec and 60 C for 1 min. All PCRs were performed in duplicate, with the mean being used to determine mRNA levels. A control containing DEPC-treated water instead of sample cDNA was included in each plate. Each set of primers generated a single PCR product of the appropriate size when visualized by agarose gel electrophoresis after qRT-PCR. Nucleotide sequences of the resultant PCR products were confirmed by BLAST ([http:// www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The amplification efficiency was determined by plotting log cDNA dilution against  $\Delta C_T$  ( $\Delta C_T = C_{T.Target} - C_{T.GAPDH}$ ), the slope of which was close to zero, indicating maximal and similar efficiency of the target and reference genes (data not shown).  $C_T$  stands for Cycle threshold and is a measurement for the number of PCR cycles (in Real-time PCR) needed to get a fluorescent signal. Relative Runx2 or N-cad mRNA levels were determined using the formula  $2^{-\Delta\Delta C_T}$  where  $\Delta\Delta C_T = (C_{T.Target} - C_{T.GAPDH})_x - (C_{T.Target} - C_{T.GAPDH})_0$ . In this formula, X represents siRNA transfection with control cultures being assigned a value of zero (Kenneth JL and Thomas DS, 2001). Data were analysed using SDS 2.0 software

(PerkinElmer Applied Biosystems). This experimental approach was further validated by the observation that differences between the  $C_T$  for the target gene and GAPDH remained relatively constant for each amount of cDNA examined.

## **2.8: Western blot analysis**

Cultures of EVT<sub>1</sub> or choriocarcinoma cell lines were washed three times in PBS and incubated in 100  $\mu$ l of cell extraction buffer (Biosource International, Camarillo, CA, USA) supplemented with 1.0 mM phenylmethylsulphonyl fluoride and protease-inhibitor cocktail for 30 min on a rocking platform. The cell lysates were centrifuged at 10 000 x g for 10 min at 4°C and the supernatants used for Western blot analysis. The concentrations of protein in the cell lysates were determined using a BCA kit (Pierce Chemicals, Rockford, IL, USA). Aliquots (approximately 30  $\mu$ g) of the cell lysates were prepared, and subjected to electrophoresis and immunoblotting, as previously described (MacCalman *et al.*, 1996) using antibodies directed against human TWIST (Santa Cruz Inc, Santa Cruz, CA, USA), RUNX2 (Santa Cruz Inc, Santa Cruz, CA, USA), E-CAD (Transduction Laboratory, Lexington, KY, USA) or N-CAD (Upstate, Lake Placid, NY, USA). To standardize the amounts of protein loaded into each lane, the blots were re probed with a polyclonal antibody directed against human  $\beta$ -actin (ACTIN) (Sigma Aldrich). The Amersham enhanced chemiluminescence system was used to detect the amount of each antibody bound to antigen with exposure to X-ray film. The absorbance values (density) obtained for TWIST, RUNX2, E-CAD or N-CAD by densitometry were then normalized relative to the corresponding ACTIN absorbance value.

## **2.9: siRNA transfection**

siRNA (Qiagen, Valencia, CA, USA; 150 ng/ 35mm<sup>2</sup> culture dish) targeting human Twist mRNA (5'-AAGAACACCTTTAGAAATAAA-3'), Runx2 mRNA (5'-CACCTTGACCATAACGTCTT-3') or N-cad mRNA (5' AAGGAGTCAG CAGAAGTTGAA-3') was transfected into BeWo or HTR-8/SVneo cells using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to a protocol outlined by the manufacturer. BeWo or HTR-8/SVneo cells transfected with a non-silencing or scrambled siRNA (5'-AAT TCT CCG AAC GTG TCA CGT-3'), served as negative controls for these studies.

Following optimization of the HiPerFect:siRNA concentration ratio, experiments were performed using BeWo or HTR-8/SVneo cells that had been transfected with either siRNA or scrambled siRNA for 0, 12, 24, 36 or 48 h.

## **2.10: Expression vector**

A full length human Twist cDNA (GenBank ID: BC036704) in pOTB7 vector was purchased from ATCC (Manassas, VA, USA). Twist cDNA was ligated into the BamH I/EcoRI site of pEF1 $\alpha$  expression vector (Invitrogen, Carlsbad, CA) using standard molecular biology techniques. A clone (pEF1 $\alpha$ -Twist) containing the Twist cDNA in the forward orientation was subsequently identified by DNA sequence analysis. Transfection reagent alone served as a control in my studies.

### **2.11: Generation of stably transfected BeWo cell line**

Stable transfections were performed to establish BeWo cell line constitutively expressing pEF1 $\alpha$ -Twist. pEF1 $\alpha$ -Twist expression vectors (2.0  $\mu$ g/ml) was transfected into BeWo cells using Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells that were successfully transfected with pEF1 $\alpha$ -Twist expression vectors were first selected after 24 h of culture using G418 antibiotic (400  $\mu$ g/ml F12K; Invitrogen). Positives were then sub-cultured by limiting dilution and expanded into a cell line that was maintained in the selection medium.

### **2.12: Indirect immunofluorescence**

Indirect immunofluorescence was performed using BeWo cells that had been plated on glass coverslips and fixed in methanol at -20°C for 2 min. Coverslips were incubated with primary antibodies for 45 min at 37°C. Primary antibodies were detected by using Alexa Fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). BeWo cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO). The coverslips were examined by using a Leica DMR microscope/ Orca Hamamatsu system and analyzed with OpenLab software (Improvision, Lexington, MA, USA). The antibodies used for indirect immunofluorescence are similar to the antibodies used in western blotting.

### 2.13: Matrigel invasion assay

Cellular invasion assays were performed by using Transwells fitted with Millipore Corp. membranes coated with a thin layer of growth factor-reduced Matrigel (6.5-mm filters, 8- $\mu$ m pore size; Costar, Toronto, ON, Canada) as previously described (Zhou *et al*, 1997; Xu *et al*, 2002). Briefly,  $2.5 \times 10^4$  cells/250  $\mu$ l of DMEM supplemented with 10 % FBS were plated in the upper wells of the Transwell invasion chambers. The Transwells were then immediately immersed into the lower wells of the invasion chambers which contained 1.2 ml of DMEM. Invasion assays were performed for 24 h in a humidified environment (5% CO<sub>2</sub>) at 37°C, after which cells attached to the porous membranes were fixed in 4% paraformaldehyde, and cells from the upper surface of the Matrigel layer were completely removed by gentle swabbing. The remaining cells that had invaded into the Matrigel and appeared on the underside of the filters were fixed and stained using a Diff-Quick Stain Kit (Dade AG, Dudingon, Switzerland) according a protocol outlined by the manufacturer. The filters were then rinsed with water, excised from Transwells, and mounted upside-down onto glass slides. Invasion indices were determined by counting the number of stained cells in 10 randomly selected, non-overlapping fields at 40x magnification using a light microscope. Each cell culture was tested in triplicate wells, on three independent occasions.

## 2.14: Statistical analysis

The absorbance values obtained from the semi-quantitative RT-PCR, real-time RT-PCR products and the fluorograms generated by Western blotting were subjected to statistical analysis using GraphPad Prism 4 computer software (San Diego, CA, USA). Statistical differences between the absorbance values were assessed by analysis of variance (ANOVA). Differences were considered significant when  $P < 0.05$ . Significant differences between the means were determined using Dunnett's test. The results are presented as the mean relative absorbance ( $\pm$  SEM) obtained from 4 different experiments.

## **CHAPTER 3: TWIST REGULATES CADHERIN-MEDIATED DIFFERENTIATION AND FUSION OF HUMAN TROPHOBLASTIC CELLS *IN VITRO***

### **3.1: Introduction and rationale**

The human placenta plays a key role in regulating growth, development, and survival of the fetus during pregnancy (Aplin, 1991). It is the site of transfer of respiratory gasses, nutrients and waste products between the maternal and fetal systems. It serves as a barrier against blood-borne pathogens and the maternal immune system. It also fulfills an endocrine role by secreting hormones, growth factors and other bioactive substances required for the establishment and maintenance of pregnancy. Upon implantation, cytotrophoblastic cells proliferate and differentiate to form syncytial trophoblasts, the outer cell layers of chorionic villi (Kaufman, 1985). Less recognized is that the syncytial trophoblast, a large multinucleated cell that forms the continuous outer layer of the human placenta, is responsible for the majority of biological functions assigned to this dynamic tissue (Richart, 1961; Kliman *et al.*, 1986).

The multinucleated syncytial trophoblast is formed from the underlying layer of mitotically active, mononucleate cytotrophoblasts, involving a cellular process dependent upon a precise series of membrane-mediated events (Douglas and King, 1990). The cadherins are likely molecular players that mediate terminal differentiation and fusion of the cytotrophoblast. They belong to a gene superfamily of integral membrane glycoproteins that mediate calcium-dependent cell adhesion through homophilic

interactions. There is a marked reduction in E-cad expression during the aggregation, differentiation and fusion of human trophoblastic cells *in vitro* (MacCalman *et al.*, 1996; Getsios *et al.*, 2000). Immunoneutralization studies have shown that an antiserum directed against the extracellular domain of E-CAD inhibits the formation of syncytial trophoblast (Coutifaris *et al.*, 1991). Taken together, these observations have led to the proposal that down-regulation of E-cad plays discrete roles in differentiation and fusion events of human trophoblastic cells *in vitro*. Although the molecular mechanisms underlying the down-regulation of E-cad during these cellular events remain to be elucidated, transcriptional repression mechanisms have emerged as one of the crucial processes for down-regulating E-cad expression during embryonic development (Carver *et al.*, 2001; Castanon and Baylies, 2002; Thiery, 2003) and tumorigenesis (Bussemakers *et al.*, 1994; Baudry *et al.*, 2003; Yang *et al.*, 2004). In particular, the highly conserved basic helix-loop-helix (bHLH) transcription factor known as TWIST has been shown to inhibit human E-cad gene expression (Yuen *et al.*, 2007; Zhang *et al.*, 2007).

Previous studies have demonstrated that down-regulation of E-cad is necessary during the terminal differentiation and fusion of human trophoblasts *in vitro* (Coutifaris *et al.*, 1991; MacCalman *et al.*, 1996; Getsios *et al.*, 2000), but none of these studies have investigated the transcriptional factors that may down-regulate E-cad during these processes *in vitro*. Taken together, it is possible that Twist is involved in the mechanisms underlying the formation and organization of the human placenta through down-regulation of E-cad. In these studies, I first determined the levels of Twist and E-cad mRNA and protein during the terminal differentiation and fusion of BeWo cells cultured



in the presence of 8-bromo-cAMP by using semi-quantitative RT-PCR and Western blotting, respectively. Next, by establishing a stably transfected cell line containing Twist cDNA or utilizing a siRNA targeting Twist, I examined whether Twist is capable of promoting the terminal differentiation in these trophoblastic cells through affecting E-cad expression. Immunofluorescence staining was used in these studies to examine the morphological changes and the localization of TWIST and E-CAD in these cells.

Dr. S. Getsios helped perform the immunofluorescence staining in Figure 3.3 and Dr. H. Zhu helped perform the semi-quantitative RT-PCR, Western blot and immunofluorescence staining in Figures 3.8 and 3.9.

## 3.2: Results

### 3.2.1: 8-bromo-cAMP promotes the terminal differentiation and fusion of BeWo cells in association with altered Twist and E-cad expression

Twist mRNA was present in cultured BeWo cells, and its levels remained relatively constant over 2 days culture in the absence of 8-bromo-cAMP (Figure 3.1 A). However, a significant increase ( $P < 0.05$ ) in Twist mRNA levels was detectable when the BeWo cells were cultured in the presence of a fixed concentration of 1.5 mM 8-bromo-cAMP for 24 h (Figure 3.1 A). Levels of Twist mRNA in these cell cultures continued to increase up to 48 h after treatment. The E-cad mRNA levels remained relatively constant in BeWo cells cultured in the absence of 8-bromo-cAMP (Figure 3.2 A). However, E-cad mRNA levels were significantly decreased in BeWo cells cultured in the presence of 1.5 mM 8-bromo-cAMP for 36 h, with levels continuing to decline up to 48 h (Figure 3.2 A).

Western blot analysis revealed the presence of a 28 kDa TWIST protein species in all BeWo cell cultures (Figure 3.1B). TWIST protein levels remained relatively constant in BeWo cells cultured in the absence of 8-bromo-cAMP (Figure 3.1 B). In agreement with the RT-PCR analysis, there was a significant increase ( $P < 0.05$ ) in TWIST levels in BeWo cells cultured in the presence of 8-bromo-cAMP (1.5 mM) for 24 h, and these levels continued to increase up to 48 h after treatment (Figure 3.1B). E-CAD protein levels remained relatively constant in BeWo cells cultured in the absence of 8-bromo-cAMP (Figure 3.2 B), but there was significant reduction in E-CAD levels in BeWo cells

cultured in the presence of 1.5 mM 8-bromo-cAMP for 36 h (Figure 3.2 B). Levels of E-CAD protein in these cell cultures continued to decrease up to 48 h after treatment.

By using indirect immunofluorescence, a low level of TWIST was found to be localized in the nuclei of mononucleate cytotrophoblasts (Figure 3.3 a) when the cells were cultured in the absence of 8-bromo-cAMP. In contrast, a higher level of TWIST immunoreactivity was found to be primarily localized in the nuclei of the syncytial trophoblast (Figure 3.3 d) 36 h after treatment with 1.5 mM 8-bromo-cAMP. Immunoreactive E-CAD was localized to areas of cell-to-cell contact when the cells were cultured in the absence of 8-bromo-cAMP (Figure 3.3b). In contrast, E-CAD immunoreactivity was distributed in a diffuse manner along the surface of the multinucleated syncytium after cells were cultured for 36 h in the presence of 1.5 mM 8-bromo-cAMP (Figure 3.3 e).

### **3.2.2: Twist siRNA inhibits the terminal differentiation and fusion of BeWo choriocarcinoma cells in the presence of 8-bromo-cAMP**

In order to repress the increase in Twist expression in BeWo cells after treatment with 1.5 mM 8-bromo-cAMP, I utilized a siRNA complementary to human Twist mRNA. Transfection of BeWo cells with this siRNA inhibited Twist mRNA and protein levels (Figures 3.4 A and B) from being up regulated after co-treatment of these cells with 1.5 mM 8-bromo-cAMP up to 48 h. In contrast, there was a significant increase in Twist mRNA and protein levels in BeWo cells transfected with a non-silencing scrambled siRNA under the same culture conditions and time frame (Figures 3.4 A and B). The

levels of E-cad mRNA and protein remained relatively constant at all time points examined in these studies when the cells were transfected with Twist siRNA (Figures 3.5 A and B). There was, however, a significant reduction in E-cad mRNA and protein levels in BeWo cells transfected with non-silencing scrambled siRNA over the same time frame (Figures 3.5 A and B).

I also assessed whether inhibiting Twist upregulation resulted in a concomitant decrease in terminal differentiation and fusion of BeWo cells in culture. When these cells were transfected with Twist siRNA in the presence of 1.5 mM 8-bromo-cAMP for 36 h, low levels of TWIST immunoreactivity were localized to the nuclei of the syncytial trophoblast (Figure 3.6 a-c). As well, E-CAD was localized to areas of cell-cell contact under the same conditions (Figure 3.6 d-f). In contrast, when BeWo cells were transfected with non-silencing, scrambled siRNA in the presence of 1.5 mM 8-bromo-cAMP for 36 h, TWIST was clearly localized to the nuclei of the syncytial trophoblast (Figure 3.7 a-c). As well, E-CAD immunostaining was reduced in the multinucleated syncytium that formed in the trophoblastic cell cultures under these conditions (Figure 3.7 d-f).

Furthermore, to determine whether BeWo cells transfected with Twist siRNA in the presence of 1.5 mM 8-bromo-cAMP remained mononucleated, I examined the distribution of desmoplakin in these cell cultures. Desmoplakin is an essential component of desmosomal junctions and has been used as a marker to determine cell boundaries in a wide variety of normal and malignant epithelial cells *in vitro*, including human trophoblasts (Douglas and King, 1990; Green and Gaudry, 2000; Getsios and MacCalman, 2003). Desmoplakin immunoreactivity was readily detectable at areas of

cell-cell contact (Figure 3.6 g-i) when BeWo cells were transfected with Twist siRNA in the presence of 1.5 mM 8-bromo-cAMP for 36 h. In contrast, desmoplakin immunoreactivity was distributed diffusely along the surface of the multinucleated syncytium when BeWo cells were transfected with non-silencing, scrambled siRNA in the presence of 1.5 mM 8-bromo-cAMP for 36 h (Figure 3.7 g-i).

### **3.2.3: pEF1 $\alpha$ -Twist promotes the terminal differentiation and fusion of BeWo cells: correlation with E-cad mRNA and protein levels**

In order to manipulate the up-regulation of Twist expression independently of cAMP, BeWo cells were stably transfected with the Twist expression vector pEF1 $\alpha$ -Twist. Following selection of transfected cells by G418 treatment (described in Materials and Methods, section 2.11), a pool of stable transfectants was maintained and used for experiments.

The increase in Twist in BeWo cells transfected with pEF1 $\alpha$ -Twist was concomitant with a reduction of E-cad mRNA and protein levels (Figures 3.8 A and B). In contrast, E-cad mRNA and protein were unchanged or minimally affected in mock transfected BeWo cells as compared to the untransfected parental cell line.

TWIST immunostaining was readily detectable in BeWo cells that were transfected with pEF1 $\alpha$ -Twist (Figure 3.9 a), and E-CAD immunostaining was diffuse along the cell boundary in the multinucleated syncytium that formed in these cell cultures (Figure 3.9 b). In contrast, TWIST was barely detectable in the nuclei of the mononucleate

cytotrophoblasts of mock transfected cells (Figure 3.9 d), while E-CAD was localized to areas of cell-cell contact in the mock transfected BeWo (Figure 3.9 e).

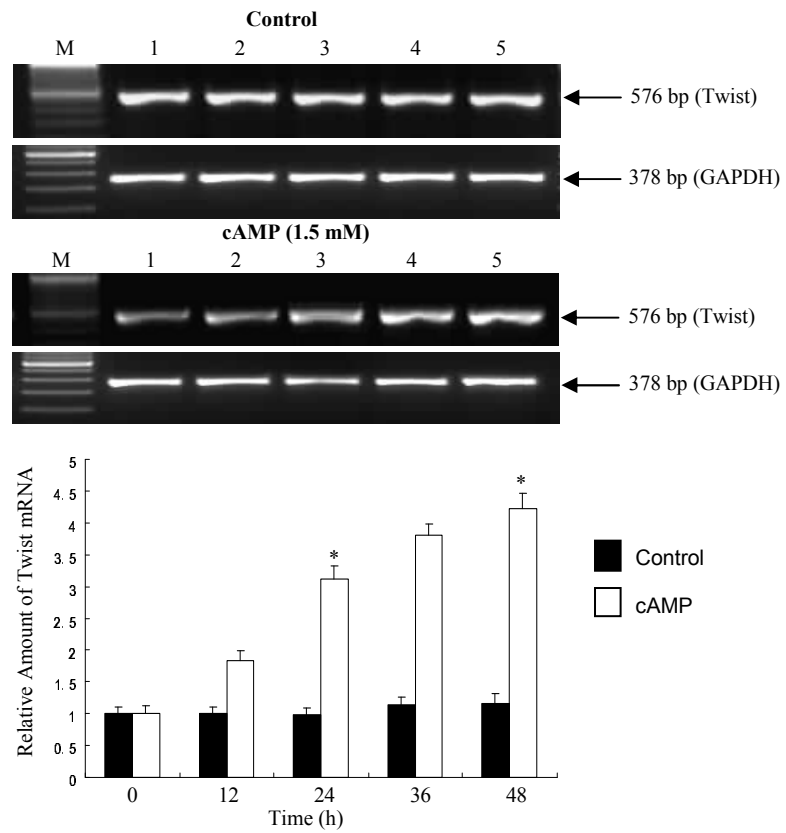
To confirm that the mononucleate BeWo cells transfected with pEF1 $\alpha$ -Twist underwent terminal differentiation and fusion to form multinucleated syncytium, I examined the distribution of desmoplakin in these cell cultures. In BeWo cells transfected with pEF1 $\alpha$ -Twist, desmoplakin immunoreactivity was diffuse along the peripheral membrane of the multinucleated syncytium that formed in these cell cultures (Figure 3.9 c). In contrast, intense desmoplakin staining was readily detectable at areas of cell-cell contact in mononucleate BeWo cells after transfection with the reagent alone (Figure 3.9 f).

Figure 3.1. Effects of cAMP on Twist mRNA and protein levels in BeWo cell cultures.

A) Semi-quantitative PCR analysis of Twist mRNA levels in BeWo cells cultured in the presence or absence (control) of a fixed concentration of 1.5 mM 8-bromo-cAMP (cAMP) for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively). A 100-bp ladder is shown in lane M (marker) with the size of the cDNA indicated at the right. The Twist mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. B) Representative fluorogram of a Western blot containing total protein (30  $\mu$ g) extracted from BeWo cells cultured in the presence or absence (control) of 1.5 mM cAMP for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively) and probed with rabbit polyclonal antibodies against TWIST or human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST protein were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN). The results from four sets of experiments were standardized to the 0 h control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to 0 h control).

Figure 3.1

A



B

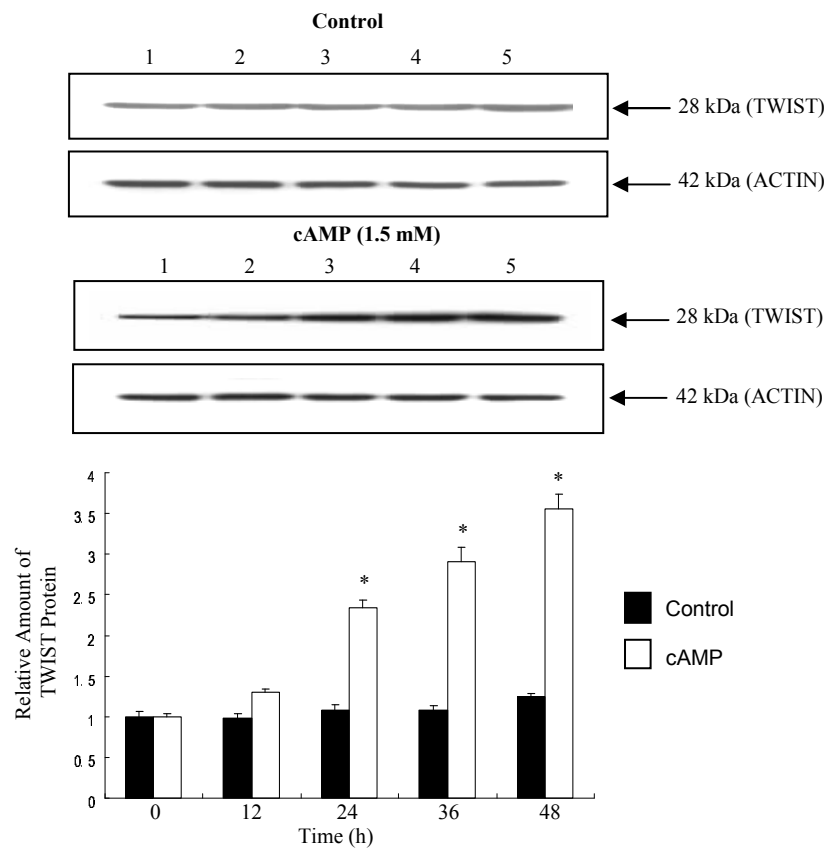


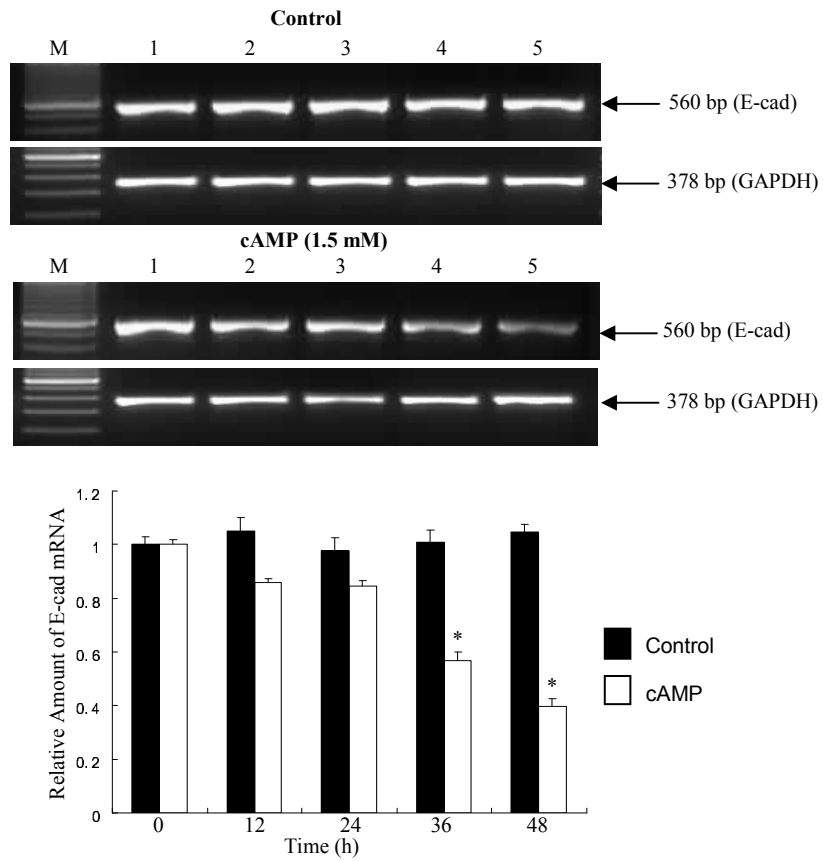


Figure 3.2. Effects of cAMP on E-cad mRNA and protein levels in BeWo cell cultures.

A) Semi-quantitative PCR analysis of E-cad mRNA levels in BeWo cells cultured in the presence or absence (control) of 1.5 mM 8-bromo-cAMP (cAMP) for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively). A 100-bp ladder is shown in lane M (marker) with the size of the cDNA indicated at the right. Values for E-cad mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from BeWo cells cultured in the presence or absence (control) of 1.5 mM cAMP for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively) and probed with a mouse monoclonal antibody directed against E-CAD. The blots were then re-probed with a polyclonal antibody specific for human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values for E-cad were normalized to the corresponding absorbance values for human  $\beta$ -actin (ACTIN). The results derived from both these analyses and from three other sets of experiments were standardized to the 0 h control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to 0 h control).

Figure 3.2

A



B

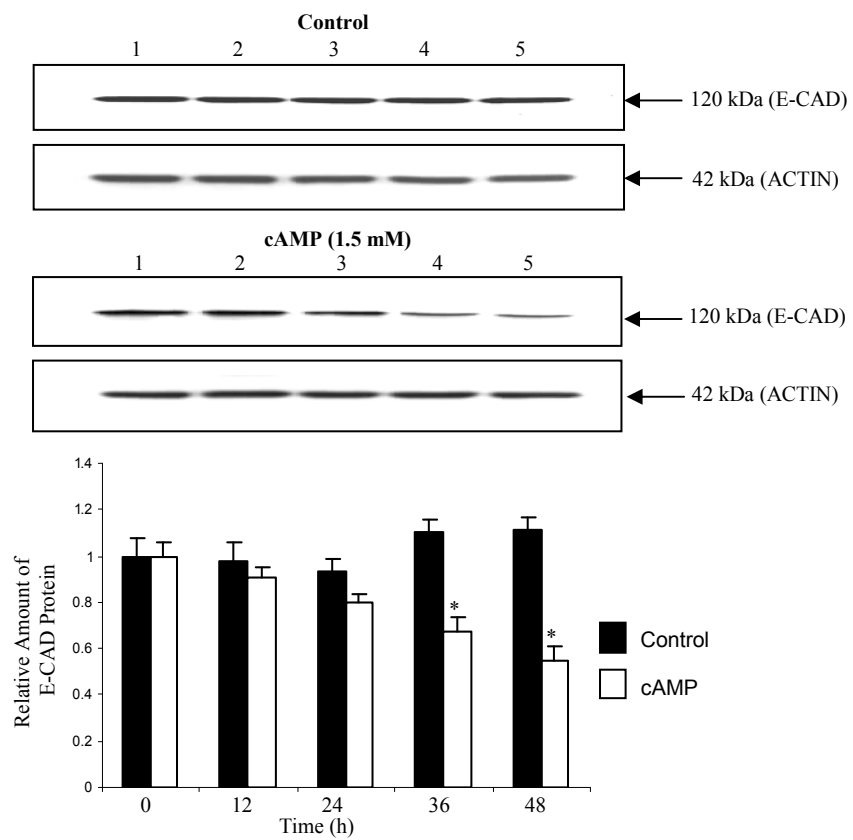


Figure 3.3. Immunolocalization of TWIST and E-CAD in BeWo cells cultured in the presence or absence (control) of 1.5 mM 8-bromo-cAMP (cAMP). Double-label immunofluorescence was carried out for BeWo cells cultured in the absence (a, b and c) or presence of 1.5 mM cAMP for 48 h (d, e and f). The cells were fixed and immunostained with a rabbit polyclonal antibody directed against TWIST (a and d) and a mouse monoclonal antibody directed against E-CAD (b and e). DAPI was used to detect the nuclei in these BeWo cell cultures (c and f). The experiment was repeated on three independent occasions. Scale bar represents 50  $\mu$ m.

Figure 3.3

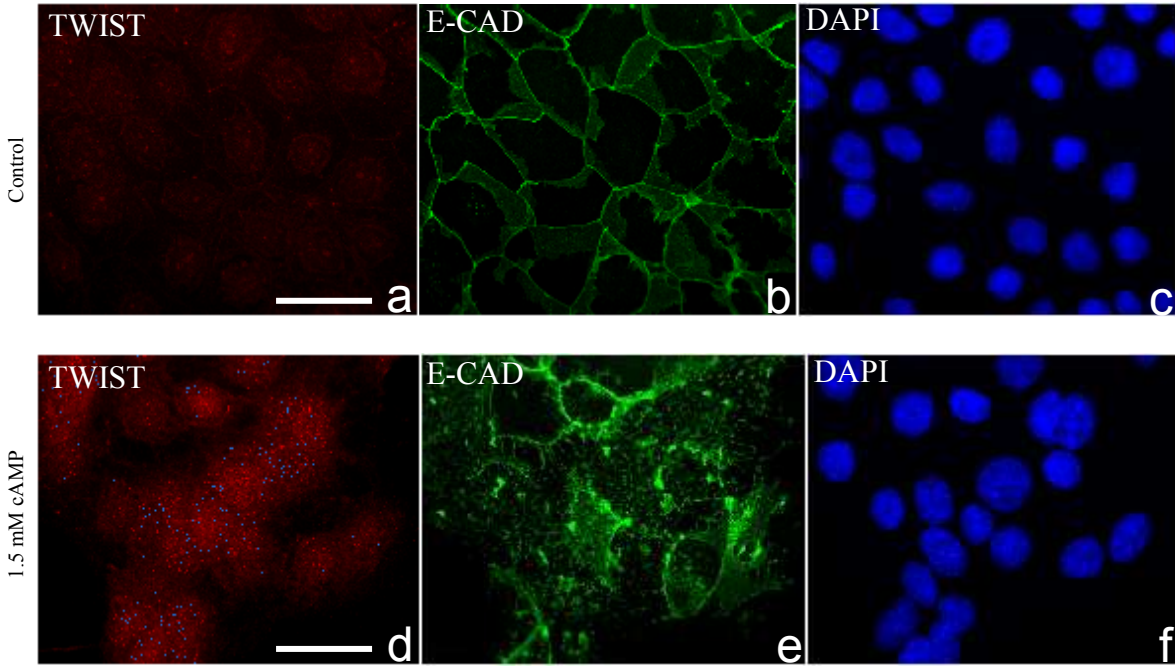
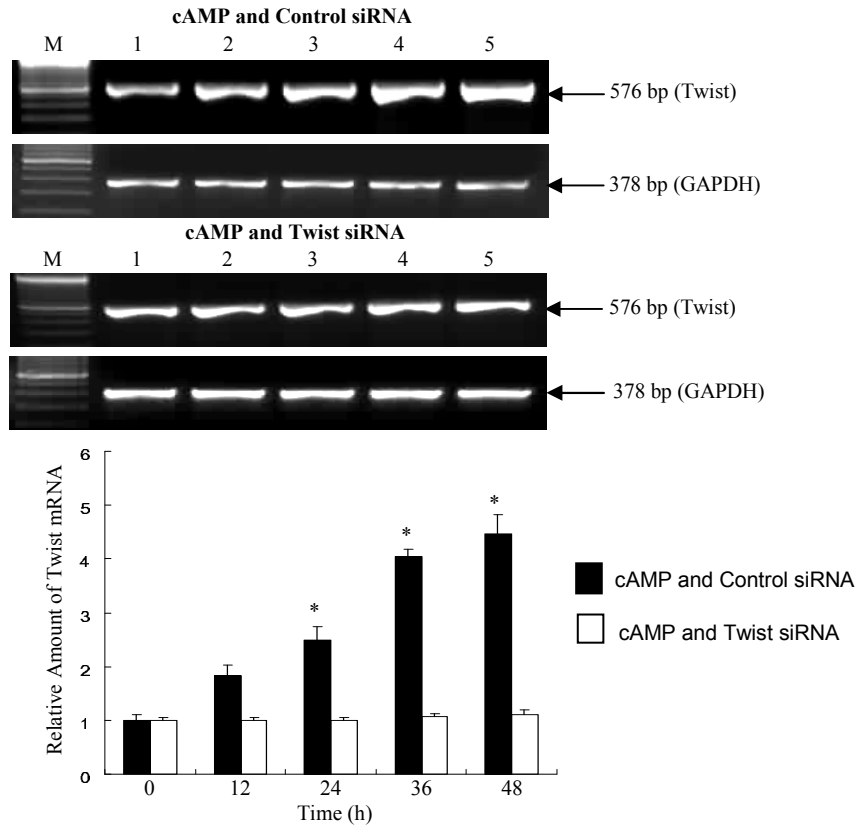


Figure 3.4. Effects of Twist siRNA on Twist mRNA and protein levels in BeWo cells cultured in the presence of 1.5 mM 8-bromo-cAMP (cAMP). A) Semi-quantitative PCR analysis of Twist mRNA levels in BeWo cells transfected with siRNA specific for Twist or a scrambled control siRNA and cultured in the presence of 1.5 mM cAMP for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively). A 100-bp ladder is shown in lane M (marker) with the size of the cDNA indicated at the right. The Twist mRNA levels in each sample were normalized against the corresponding GAPDH mRNA levels. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from BeWo cells cultured after transfection with siRNA specific for Twist or a scrambled control siRNA in the presence of a 1.5 mM cAMP for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively), and probed with rabbit polyclonal antibodies directed against TWIST or human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values for TWIST were normalized to the absorbance values for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results from these analyses and from three other sets of experiments were standardized to the 0 h control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to 0 h control).

Figure 3.4

A



B

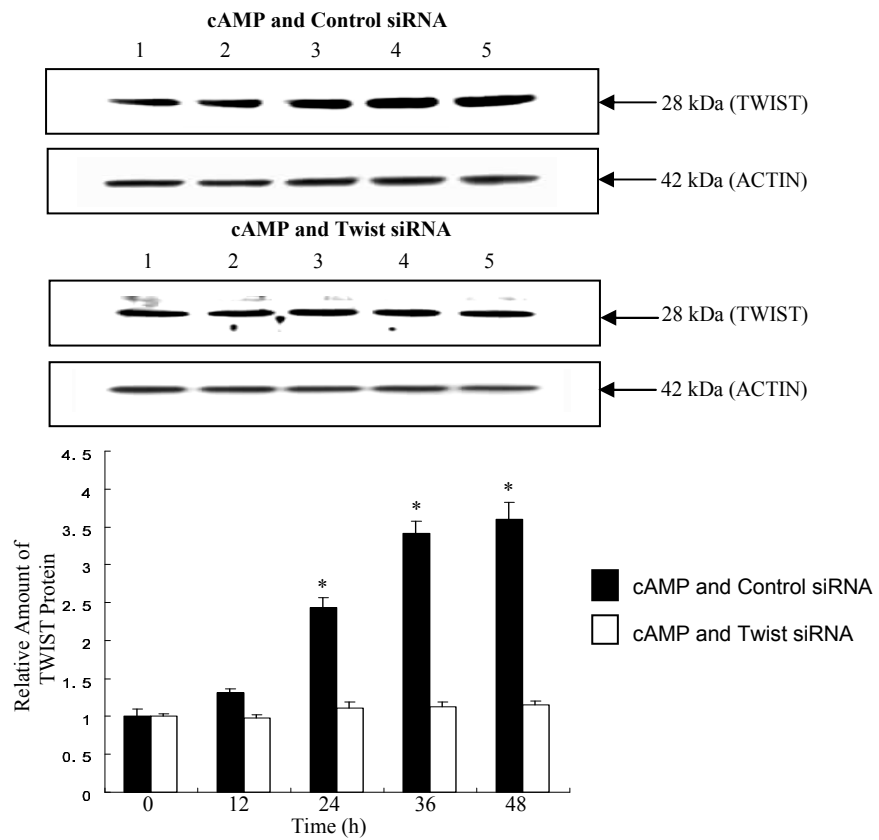


Figure 3.5. Effects of Twist siRNA on E-cad mRNA and protein levels in BeWo cells cultured in the presence of 1.5 mM 8-bromo-cAMP (cAMP). A) Semi-quantitative PCR analysis of E-cad mRNA levels in BeWo cells transfected with siRNA specific for Twist or a scrambled control siRNA and cultured in the presence of 1.5 mM cAMP for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively). A 100-bp ladder is shown in lane M (marker) with the size of the cDNA indicated to the right. The E-cad mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from BeWo cells cultured after transfection with siRNA specific for Twist or a scrambled control siRNA in the presence of a fixed concentration of cAMP (1.5 mM) for 0, 12, 24, 36, or 48 h (lanes 1-5, respectively), and probed with mouse monoclonal antibody directed against E-CAD or polyclonal antibody against human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for E-CAD were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in corresponding samples. The results from these analyses and from three other sets of experiments were standardized to the 0 h control, and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to 0 h control).

Figure 3.5

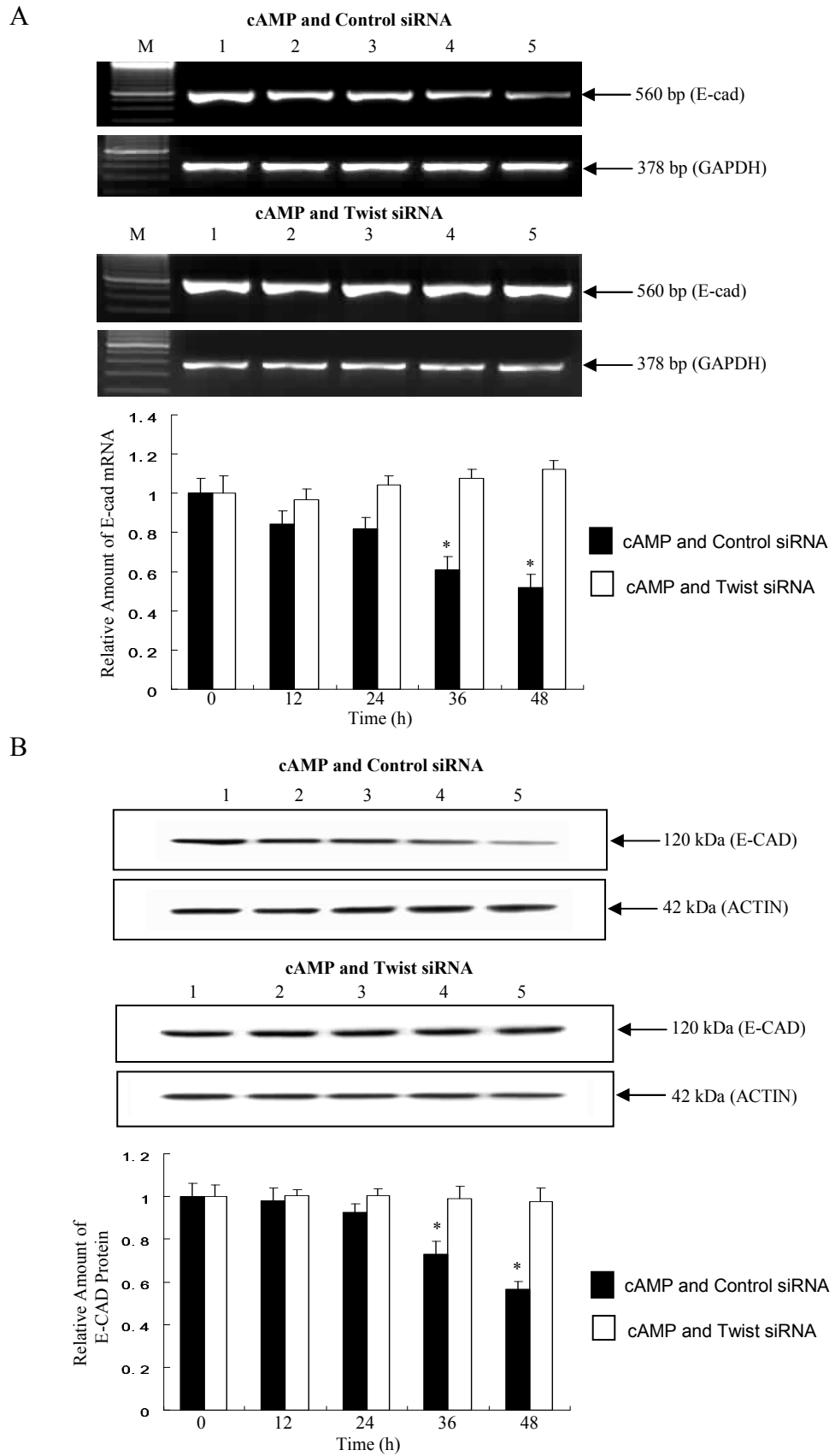




Figure 3.6. Immunolocalization of TWIST, E-CAD and desmoplakin (DESMOPLAKIN) in BeWo cells transfected with siRNA specific for Twist in the presence of 1.5 mM 8-bromo-cAMP (cAMP). Photomicrographs of immunoreactive TWIST (a), E-CAD (d) and desmoplakin (g) expression in BeWo cells transfected with siRNA specific for Twist and cultured in the presence of 1.5 mM cAMP for 36 h. The cells were fixed and immunostained with either a rabbit polyclonal antibody against TWIST or a monoclonal antibody against E-CAD. A rabbit polyclonal antibody against desmoplakin was used as a marker for cell-cell borders. DAPI was used to detect the nuclei of these BeWo cells (b, e and h). Merged signals are shown on the right (c, f and i). The experiment was repeated on three independent occasions. Scale bar represents 50  $\mu$ m.

Figure 3.6

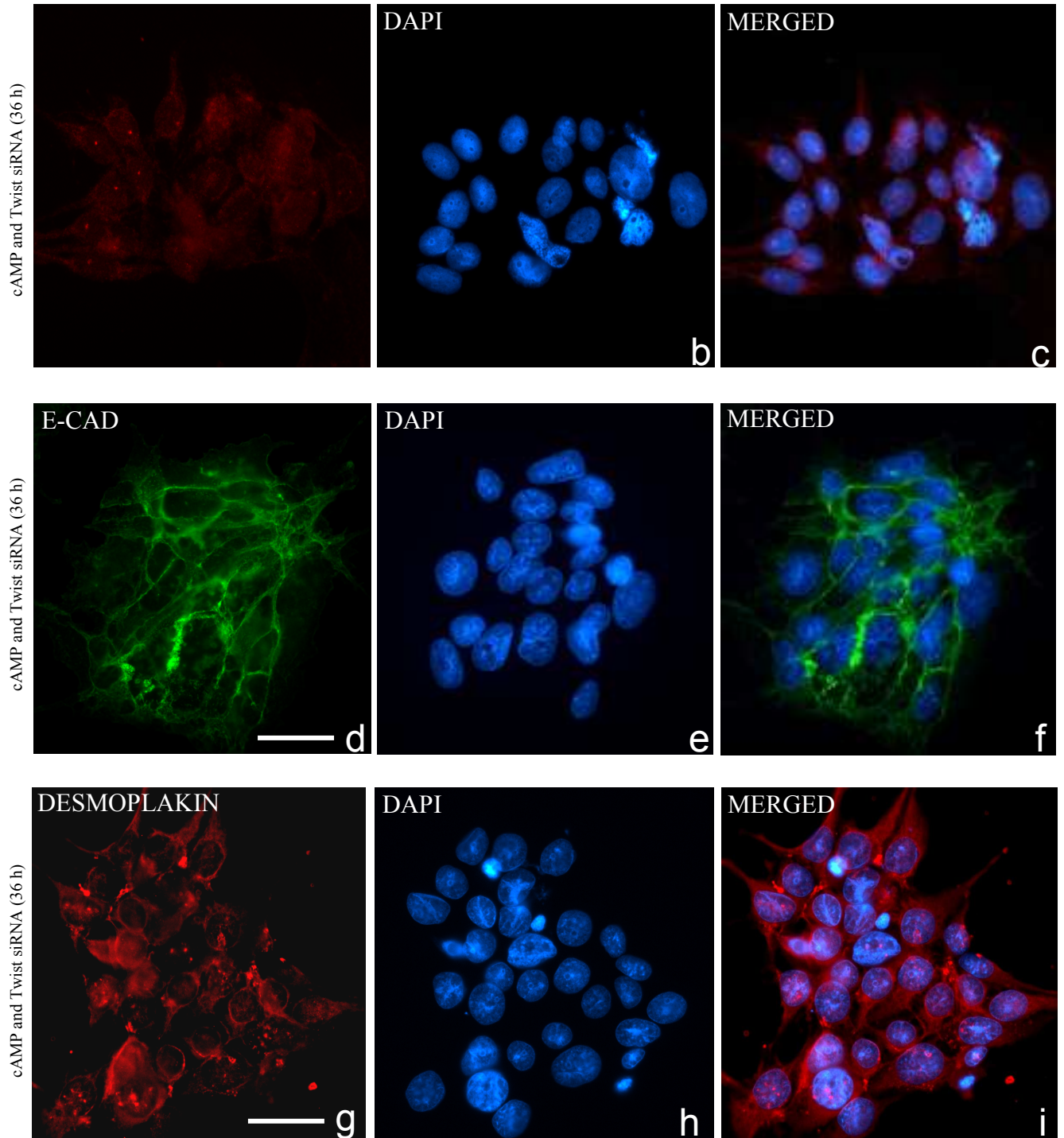


Figure 3.7. Immunolocalization of TWIST, E-CAD and desmoplakin (DESMOPLAKIN) in BeWo cells transfected with a scrambled control siRNA in the presence of 1.5 mM 8-bromo-cAMP (cAMP). Photomicrographs of immunoreactive TWIST (a), E-CAD (d) and desmoplakin (g) in BeWo cells transfected with a scrambled control siRNA and cultured in the presence of 1.5 mM cAMP for 36 h. The cells were fixed and immunostained with either a rabbit polyclonal antibody against TWIST, a monoclonal antibody against E-CAD, or a rabbit polyclonal antibody against desmoplakin. DAPI was used to detect the nuclei of these BeWo cells (b, e and h). Merged signals are shown on the right (c, f and i). The experiment was repeated on three independent occasions. Scale bar represents 50  $\mu\text{m}$ .

Figure 3.7

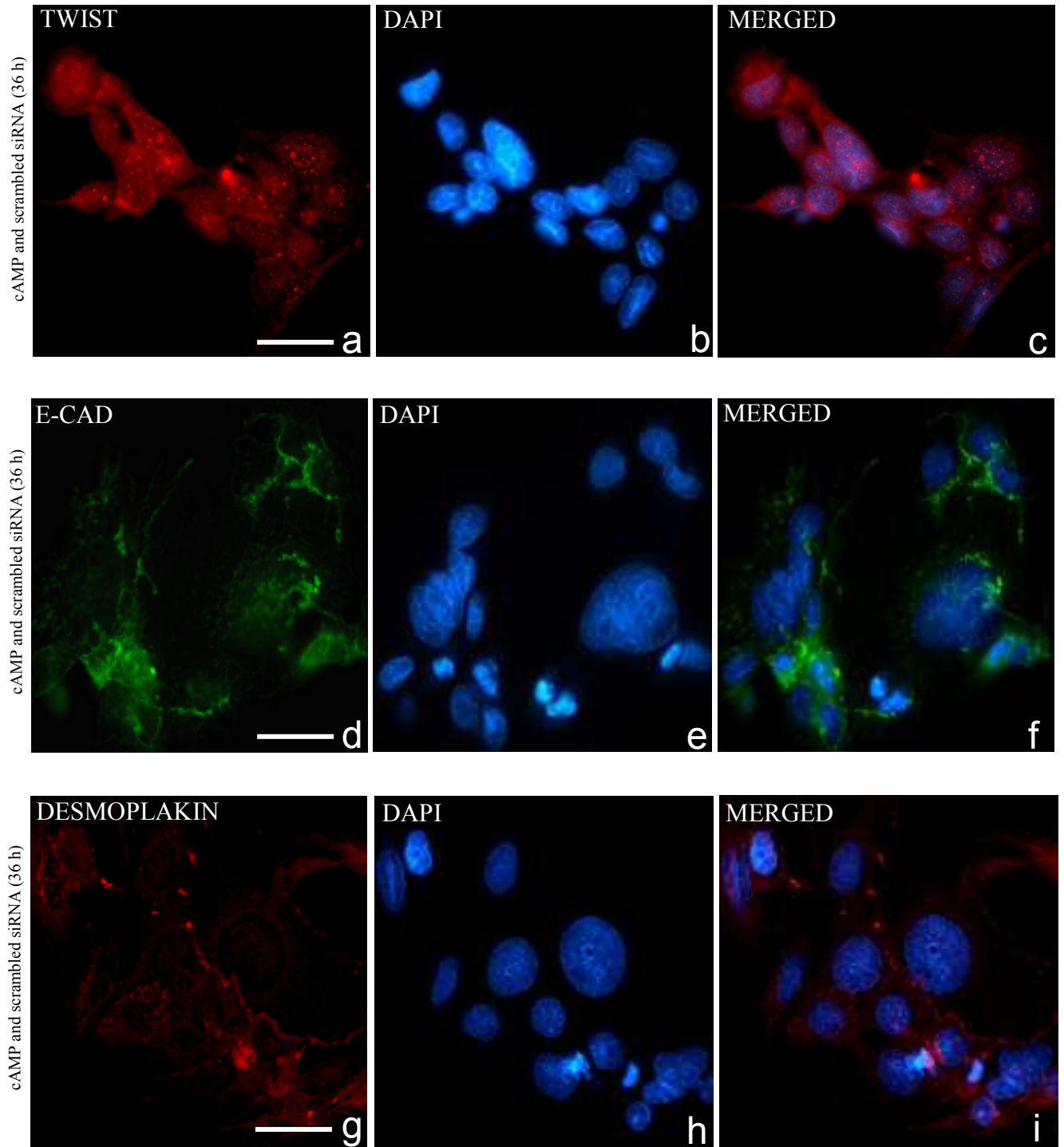


Figure 3.8. Twist and E-cad mRNA and protein levels in BeWo choriocarcinoma cells stably transfected with pEF1 $\alpha$ -Twist. A) Semi-quantitative PCR analysis of Twist or E-cad mRNA levels in untransfected BeWo cells (Wild), mock transfected BeWo cells (Reagent), or BeWo cells transfected with pEF1 $\alpha$ -Twist (pEF1 $\alpha$ -Twist). Values for Twist or E-cad mRNA levels in each sample were normalized to their corresponding GAPDH mRNA levels. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from untransfected BeWo cells (Wild), mock transfected BeWo cells (Reagent), or BeWo cells transfected with pEF1 $\alpha$ -Twist (pEF1 $\alpha$ -Twist) and probed with specific antibodies directed against TWIST or E-CAD. The blots were then re-probed with a polyclonal antibody specific for human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST or E-CAD were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in corresponding samples. The results from these analyses and from three other sets of experiments were standardized to the values of the transfection reagent control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to control).

Figure 3.8

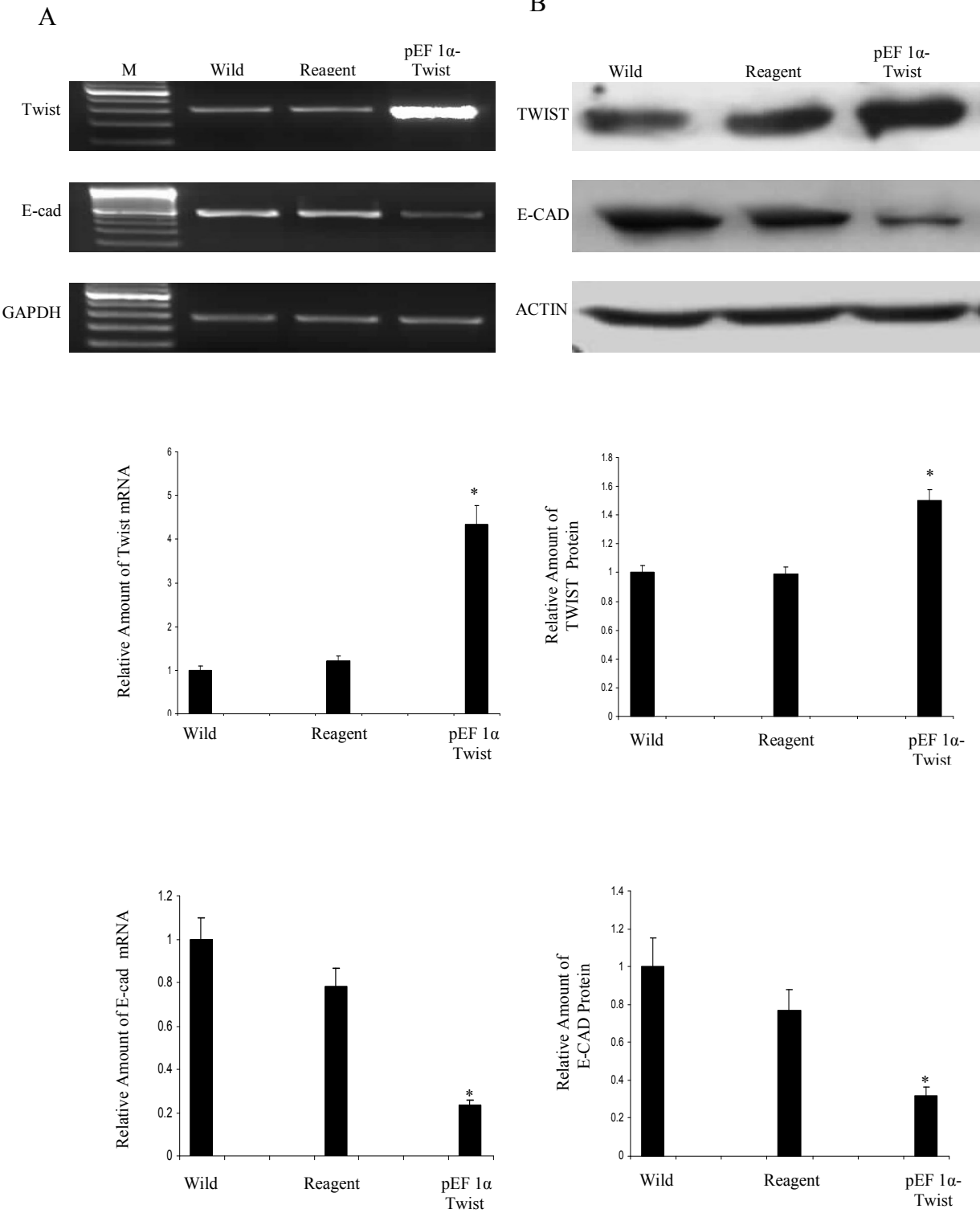
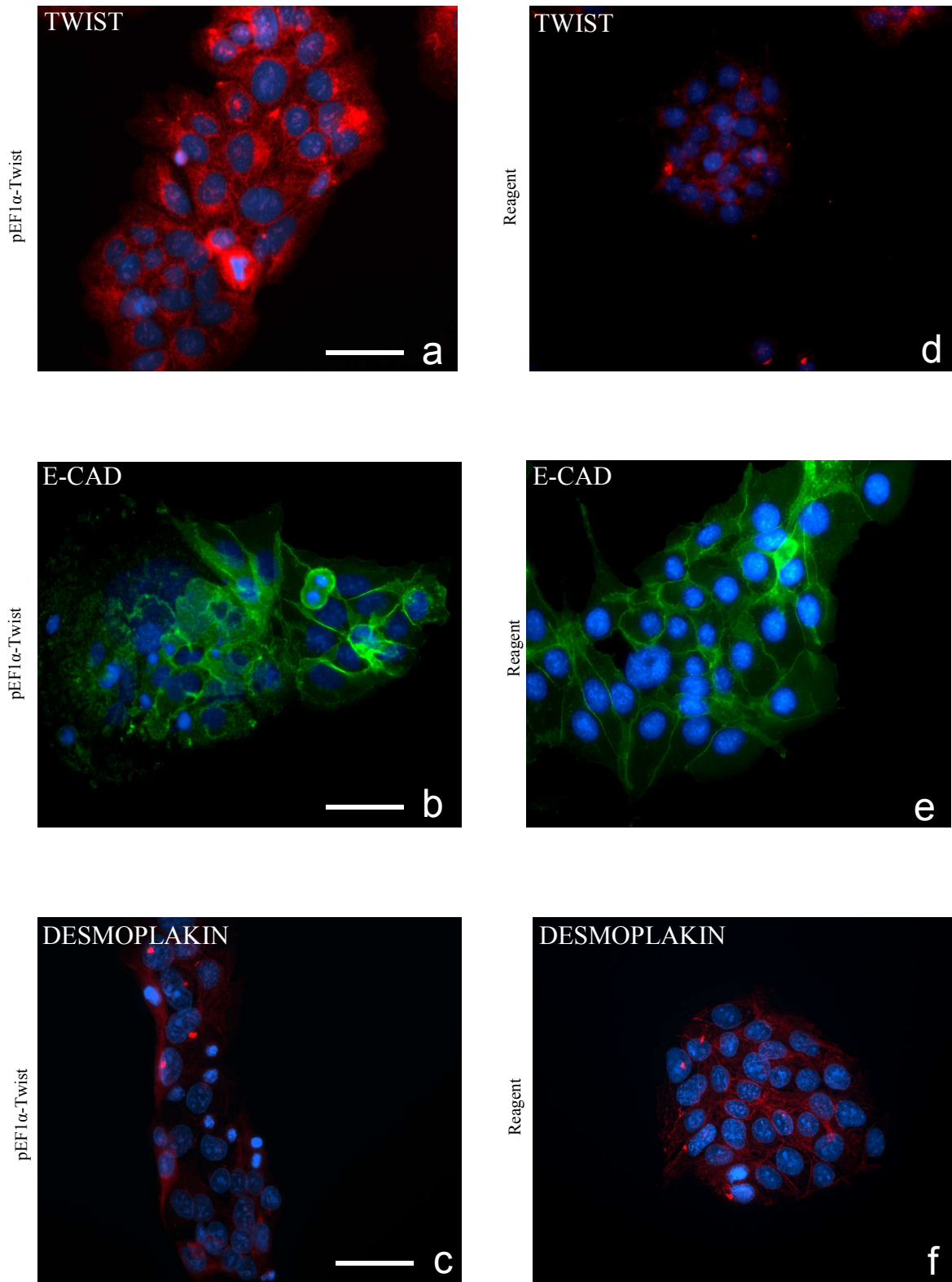


Figure 3.9. Immunolocalization of TWIST, E-CAD and desmoplakin (DESMOPLAKIN) in BeWo cells stably transfected with pEF1 $\alpha$ -Twist (pEF1 $\alpha$ -Twist) or in mock transfected BeWo cells (Reagent). The Twist overexpressing cells (a-c) or the mock transfected cell (d-f) were fixed and immunostained with either a rabbit polyclonal antibody against TWIST (a and d), a mouse monoclonal antibody against E-CAD (b and e), or a rabbit polyclonal antibody against desmoplakin (c and f). DAPI was used to detect the nuclei in these BeWo cell cultures. The experiment was repeated on three independent occasions. Scale bar represents 50  $\mu$ m.

Figure 3.9





### 3.3: Discussion and summary

Twist was first identified in *Drosophila melanogaster* as one of the zygotic genes required for dorsoventral patterning and mesoderm differentiation during embryogenesis (Thisse *et al.*, 1987; Gitelman, 1997). This transcription factor is recognized as an organizer of epithelial-mesenchymal transition (EMT) during gastrulation and regulator of mesoderm differentiation (Thisse *et al.*, 1987; Leptin *et al.*, 1990). Twist has also been found to play an important role in cancer metastasis and was first reported in a breast cancer model, which suggested that Twist induced EMT and resulted in the promotion of tumour invasion (Yang *et al.*, 2004). Similarly, disruption of E-CAD-mediated cell adhesion seems to be crucial in the EMT from non-invasive to invasive tumour cells (Comijn *et al.*, 2001; Yang *et al.*, 2004; Lee *et al.*, 2006). Also, disruption of E-CAD-mediated cell adhesion has been related to a more infiltrative growth pattern in different types of cancers (Sakuragi *et al.*, 1994; Cheng *et al.*, 1996; Bremnes *et al.*, 2000). In agreement, high Twist expression was seen to be correlated with deep myometrial invasion by endometrial cancer cells and is concurrent with decreased E-cad expression (Kyo *et al.*, 2006).

In my own studies, I observed a differential expression of Twist and E-cad during the terminal differentiation and fusion of a human trophoblastic cell line *in vitro*. These results suggest that TWIST controls the expression of E-cad, and that together they play an important role in human placental development. Importantly, the finding that Twist is up-regulated and E-cad is down-regulated when BeWo cells undergo differentiation and

fusion to become syncytia, suggests that TWIST may serve as a transcription repressor of E-cad during this highly regulated series of membrane-mediated processes.

My observation of E-cad down-regulation during Twist-regulated differentiation and fusion of human trophoblastic cell line *in vitro* is consistent with other reports that loss of E-cad expression is a critical process during human trophoblast differentiation (Coutifaris *et al.*, 1991; Getsios *et al.*, 2003). For example, E-CAD has been shown to be present on the surface of cytotrophoblasts *in situ*, but not on the surface of the encompassing syncytiotrophoblast (Eidelman *et al.*, 1989). Furthermore, the loss of E-CAD function by function-perturbing antibodies against E-CAD disrupted the aggregation of mononucleate cytotrophoblasts isolated from the human term placenta, which in turn inhibited the formation of multinucleated syncytia in cell cultures (Coutifaris *et al.*, 1991).

Primary trophoblast cultures were not used in my studies mainly because these samples contain heterogeneous populations of isolated cyto- and syncytial trophoblasts (Nasiry *et al.*, 2006). To circumvent this problem, I used the fusigenic BeWo choriocarcinoma cell line, which has long been known to respond to increased intracellular cAMP by differentiating into a multinucleated syncytial trophoblast (Pattillo and Gey, 1968; Wice *et al.*, 1990).

Here, I also demonstrate that siRNA directed against Twist disrupts the formation of a multinucleated syncytium in BeWo cells undergoing terminal differentiation and fusion. My gain-of-function studies showed that heterologous Twist overexpression promotes the formation of multinucleated syncytia in these cell cultures. Collectively, and since E-cad expression was inversely altered in response to enhanced or reduced Twist expression, both these studies provide further evidence that E-CAD-mediated differentiation and

fusion of human trophoblastic cells are regulated by Twist. My results have also shown that terminal differentiation and fusion of human trophoblastic cells are accurately reproduced in culture at both the morphological and molecular levels.

Suppression of Twist expression in highly metastatic mammary carcinomas or prostate cancer cells inhibits their ability to invade or metastasize (Hoek *et al.*, 2004; Yang *et al.*, 2004; Kwok *et al.*, 2005; Hosono *et al.*, 2007). On the other hand, elevated levels of Twist mRNA are associated with malignant transformation of melanoma cells, increase the risk for recurrence and for poor survival in epithelial ovarian carcinoma patients, and induce mesenchymal components and facilitate cell motility of various tumour cells (Kang and Massague, 2004; Yang *et al.*, 2006; Hosono *et al.*, 2007). The major functions of E-CAD are to mediate cell-cell adhesion and to play a pivotal role in the formation and maintenance of many epithelial tissues (Suzuki *et al.*, 1996). Changes in cellular adhesion molecules like E-CAD are important for the invasive and metastatic capacity of human cancers (Takeichi M, 1993; Wijnhoven *et al.*, 2000). For instance, decreased membranous immunoreactivity of E-CAD has also been shown to predict lymph node metastasis in atypical carcinoids (Pelosi *et al.*, 2005). In contrast, increased cytoplasmic immunoreactivity of E-CAD has been suggested to result in loss of cell polarity and differentiation in pancreatic intraepithelial neoplasia (Al-Aynati *et al.*, 2004).

Several studies have strongly suggested that transcription repression is a major mechanism leading to decreased E-cad expression (Schipper *et al.*, 1991; Bussemakers *et al.*, 1992; Brabant *et al.*, 1993; Dorudi *et al.*, 1993). This commonly involves silencing of E-cad transcription through E-boxes in its promoter region. In transient reporter assays, over-expression of Twist in human mammary epithelial cells inhibited E-cad

promoter activity (Yang *et al.*, 2004). TWIST represses transcription from the E-cad promoter through the E-box sequence, 5'-CANNTG-3', which is also targeted by SNAIL and SIP1 (Lee *et al.*, 2006).

Other ways of silencing E-cad gene expression have been recognized, including gene truncation mutations and loss of heterozygosity (LOH) (Yoshiura *et al.*, 1995, Berx *et al.*, 1996; Huiping *et al.*, 1999; Droufakou *et al.*, 2001). However, these mechanisms appear to be utilized in only a subset of E-cad-negative invasive lobular carcinomas, therefore suggesting that a transcriptional repression mechanism of the E-cad promoter by TWIST plays a major role in the pathogenesis of these tumours (Yang *et al.*, 2004). Although several studies have shown that promoter methylation can influence E-cad expression (Yoshiura *et al.*, 1995; Xue *et al.*, 2003; Lombaerts *et al.*, 2006), this has not always been found (Tamura *et al.*, 2000), and the mechanisms responsible for down-regulation of E-cad may be cell type-specific.

The function of bHLH transcription factors like TWIST relies on the basic DNA-binding region and the HLH structure that allows monomers to form functional dimers that can identify and bind to the E-box DNA motif (Massari and Murre, 2000). The basic domain mediates the interaction with DNA (Elleberger *et al.*, 1994; Ma *et al.*, 1994) and bHLH proteins bind as dimers to the consensus hexanucleotide sequence E-box, (Ephrussi *et al.*, 1985). TWIST forms both homodimers (T/T) and heterodimers with E2A E proteins (T/E) (Connerney *et al.*, 2006). It has been suggested that TWIST heterodimers function as a transcription repressor, and that TWIST homodimers up-regulate expression of the target gene by functioning as a transcription activator (Castanon *et al.*, 2001; Connerney *et al.*, 2006). However, it remains to be determined if

the interaction of TWIST with these E-boxes occurs directly, *via* interactions with E2A proteins or *via* indirect mechanisms (Lee *et al.*, 2006). In *Drosophila*, Twist can increase the expression of Snail, a known repressor of E-cad transcription (Ip *et al.*, 1992). However, Twist expression fails to induce Snail in human mammary epithelial cells that had undergone EMT (Yang *et al.*, 2004). This agrees with the observation that TWIST and SNAIL function independently in mice (Carver *et al.*, 2001; Soo *et al.*, 2002).

In summary, I have determined that Twist is capable of regulating E-CAD-mediated differentiation and fusion of human trophoblastic cells *in vitro*, and these results have contributed to a new understanding of TWIST's function as a transcriptional repressor during terminal differentiation and fusion of human trophoblasts (Figure 3.10). Furthermore, my studies suggest that TWIST and E-CAD may serve as novel molecular markers for early detection of potential pregnancy disorders such as pre-eclampsia, intrauterine growth restriction (IUGR) and miscarriage.

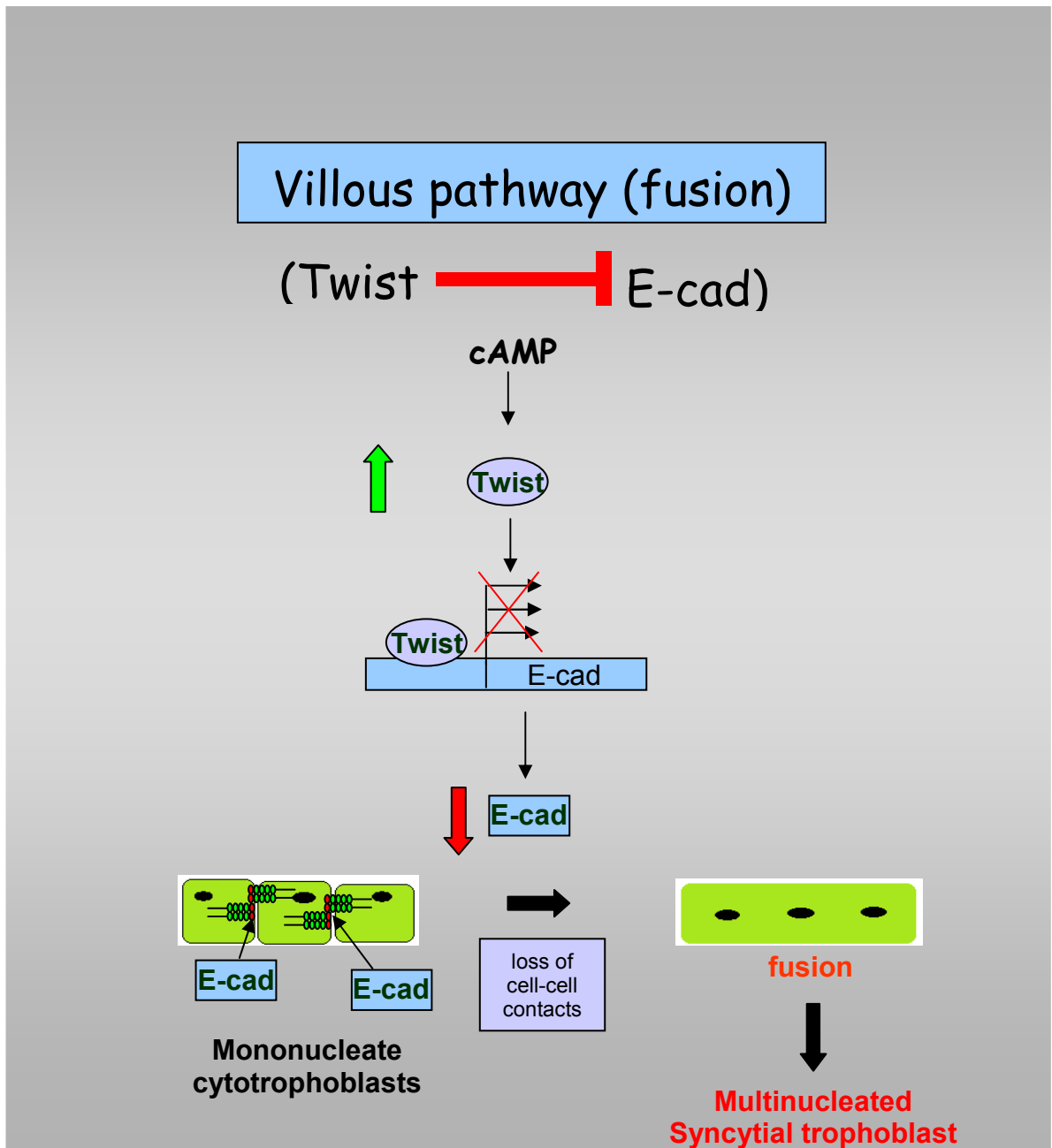


Figure 3.10. A schematic diagram of a proposed role of Twist in regulating E-cad-mediated terminal differentiation and fusion of human trophoblastic cells. cAMP upregulates TWIST levels. TWIST down-regulates E-cad expression to mediate the formation of multinucleated syncytial trophoblast from mononucleate cytotrophoblasts.

## **CHAPTER 4: TWIST REGULATES CADHERIN-MEDIATED INVASION OF HUMAN TROPHOBLASTIC CELLS *IN VITRO***

### **4.1: Introduction and rationale**

Successful implantation depends on the differentiation of mononucleate cytotrophoblasts *via* two distinct and mutually exclusive pathways. The villous cytotrophoblastic cells will proliferate and differentiate by fusion to form the outer syncytial trophoblast; or enter the extravillous pathway to form highly invasive extravillous cytotrophoblasts (EVTs) (Bischof and Campana, 2000). In the extravillous pathway, these cells invade deeply into the underlying maternal tissues (Pijnenborg *et al.*, 1980). EVTs invade the uterine stroma and superficial myometrium as individual mononucleate cells, penetrate the basal lamina, and replace the endothelia of uterine vasculature. This allows an increase in blood supply to the placenta and ensures an adequate supply of oxygen and nutrients to the developing fetus, a critical step in human pregnancy (Aplin., 1991; Pijnenborg *et al.*, 1983 and 1994). Failure of this process is associated with clinical pathological conditions such as miscarriage, intrauterine growth retardation, or preeclampsia (King and Loke, 1994). The process of human trophoblast invasion utilizes similar molecular mechanisms as those of tumour cell invasion, albeit trophoblast invasion is more tightly regulated (Lala *et al.*, 2002).

The precise control of trophoblastic cell differentiation along the extravillous pathway has been demonstrated to occur through regulated changes in cell-cell and cell-matrix interactions, and the modification of distinct extracellular matrix (ECM) components

through proteolytic degradation and/or activation (Lala and Hamilton, 1996; MacCalman et al., 1998; Chakraborty *et al.*, 2002).

To date, the molecular mechanisms that regulate trophoblast differentiation and invasion during formation and organization of the human placenta remain to be elucidated. TWIST, a highly conserved basic helix-loop-helix (bHLH) transcription factor, is known to play a key role in promoting tumour metastasis and is associated with potent invasiveness and poor prognosis of epithelial cancer (Thiery, 2002; Kang and Massague, 2004; Vernon *et al.*, 2004; Yang *et al.*, 2004; Kwok *et al.*, 2005; Lee *et al.*, 2006). In addition, Twist mRNA levels were found to be up-regulated in the highest grade of gliomas (Elias *et al.*, 2005). TWIST has also been shown to be a key regulator of N-cad expression in different types of cancer cell lines (Alexander *et al.*, 2006; Rosivatz *et al.*, 2002). TWIST is essential for the initiation of N-cad expression in *Drosophila* (Oda *et al.*, 1998).

N-CAD is a member of the superfamily of integral membrane glycoproteins that mediate calcium-dependent cell adhesion (Takeichi, 1995; Suzuki, 1996). During cancer progression, there is an increase in expression of N-cad (Tomita *et al.*, 2000; Derycke *et al.*, 2004; Hazan *et al.*, 2004). Other studies have shown a functional role for N-CAD in promoting an invasive phenotype. For example, exogenous expression of N-cad in breast epithelial cells and squamous epithelial cells results in a more invasive phenotype (Islam *et al.*, 1996; Nieman *et al.*, 2000). To date, N-cad has received significant attention in cancer studies (Hazan *et al.*, 2000).

Based on these observations, I hypothesize that Twist plays a key role in trophoblastic cell invasion through regulation of N-cad expression during human pregnancy. In my



studies, I first determined the expression levels of Twist and N-cad in the villi of first trimester human placentas, in cultures of highly invasive EVT<sub>s</sub>, and in two choriocarcinoma cell lines (JEG-3 and BeWo) by using semi-quantitative RT-PCR and Western blotting. Next, by using a Matrigel invasion assays, I determined the ability of interleukin (IL)-1 $\beta$  and transforming growth factor (TGF)- $\beta$ 1, two cytokines that are spatiotemporally expressed at the maternal-fetal interface (Graham *et al.*, 1991 and 1993), to regulate trophoblastic cell invasion and Twist expression in these cells. Loss-of-function studies using siRNA for Twist were employed to determine the role of Twist or N-cad in trophoblastic cell invasion. Finally, by using a function-perturbing antibody directed against N-CAD, the role of N-cad in regulating the invasive phenotype of these cells was assessed.

## **4.2: Results**

### **4.2.1: Determining the levels of Twist and N-cad mRNA and protein levels in the human placenta, highly invasive EVT<sub>s</sub>, and poorly invasive trophoblastic cell lines.**

Semi-quantitative RT-PCR and Western blot analysis show that Twist was expressed in the first trimester human placenta and highly expressed in EVT<sub>s</sub> propagated from first trimester human placenta (refer to Section 2.2 in Materials and Methods for preparation details) and HTR-8/SVneo, a human EVT cell line. In contrast, Twist mRNA and proteins levels were significantly lower in poorly invasive JEG-3 and BeWo trophoblastic cell lines (Figure 4.1A and B).

I then determined the expression of N-cad in human placental tissue, EVT<sub>s</sub>, and trophoblastic cell lines. N-cad expression was absent in the first trimester human placenta. It was barely detectable at the mRNA level and absent at the protein level in poorly invasive JEG-3 and BeWo trophoblastic cell lines. In contrast, N-cad mRNA and protein levels were higher significantly in EVT<sub>s</sub> and HTR-8/SVneo cells (Figure 4.2A and B).

### **4.2.2: IL-1 $\beta$ and TGF- $\beta$ 1 respectively promote and restrain the invasive ability of EVT primary cultures.**

Previous studies have shown that IL-1 $\beta$  and TGF- $\beta$ 1 play major regulatory roles in the establishment of pregnancy (Graham and Lala, 1991; Chakraborty *et al.*, 2002). In view

of these observations, I examined the ability of these two cytokines to regulate the invasive ability of EVT primary cultures.

My results show that the addition of a vehicle (ethanol) to the culture medium of EVTs had no significant effect on the invasiveness of these cells, but a significant increase in EVT invasion was observed when these cells were treated with IL-1 $\beta$  (Figure 4.3A). In contrast, TGF- $\beta$ 1 significantly reduced the invasive ability of these cells (Figure 4.3B).

I also examined the effect of TGF- $\beta$ 1 on HTR-8/SVneo cell invasion, but found that these cells did not respond at all to TGF- $\beta$ 1 treatment (data not shown).

#### **4.2.3: Time-dependent effects of IL-1 $\beta$ and TGF- $\beta$ 1 on Twist mRNA and protein levels in EVTs**

Significant increases in Twist mRNA and protein levels ( $P < 0.05$ ) were detected in primary EVTs cultured in the presence of 100 IU IL-1 $\beta$  for 24 h that were maintained or even slightly elevated after 48 h culture (Figure 4.4A and B). In contrast, the addition of TGF- $\beta$ 1 to the culture medium of these primary cells caused a significant decrease in Twist mRNA and protein levels after 24 h. Levels of Twist mRNA and protein expression continued to decrease until 48 h after treatment with this cytokine (Figure 4.5A and B).

#### **4.2.4: Concentration-dependent effects of IL-1 $\beta$ and TGF- $\beta$ 1 on Twist mRNA and protein levels in EVT $s$**

A significant increase ( $P < 0.05$ ) in Twist mRNA and protein levels was detected in primary EVT $s$  cultured for 24 h in the presence of 100 and 1000 IU IL-1 $\beta$ , with 1000 IU of IL-1 $\beta$  having the greatest effect (Figure 4.6A and B).

Twist mRNA and protein expression levels were significantly decreased in EVT $s$  cultured in the presence of TGF- $\beta$ 1 (5 and 10 ng/ml) for 24 h, but not at lower concentrations of this cytokine utilized with these primary cell cultures (Figure 4.7A and B).

#### **4.2.5: Decreased Twist down-regulates N-cad expression and reduces the invasive capacity of HTR-8/SVneo cells**

I utilized siRNA complementary to human Twist mRNA to decrease Twist expression in cultures of the HTR-8/SVneo EVT cell line. As a control, HTR-8/SVneo cells were transfected with a non-silencing (NS) scrambled siRNA. Transfection of HTR-8/SVneo cells with Twist siRNA significantly decreased Twist (Figure 4.8A and B) and N-cad (Figure 4.9A and B) mRNA and protein levels in these cell cultures after 36 h when compared to the control treatments.

I next determined whether a reduction in Twist expression in HTR-8/SVneo resulted in a concomitant decrease in their invasive capacity. I performed invasion assays using Matrigel-coated Transwell chambers. My results show that the numbers of cells that penetrated the Matrigel barrier and appeared on the underside of the Millipore filter were

significantly lower in cultures of HTR-8/SVneo transfected with Twist siRNA than in cultures transfected with scrambled control siRNA (Figure 4.10).

#### **4.2.6: Loss of N-cad reduces the invasive capacity of HTR-8/SVneo cells**

In order to directly reduce the N-cad mRNA and protein levels in HTR-8/SVneo cells, these cells were transfected with siRNA specific for N-cad. To assess siRNA efficacy, real-time RT-PCR and Western blot analysis were performed. The results demonstrated a significant reduction in N-cad mRNA and protein levels (Figure 4.11A and B) compared to HTR-8/SVneo cells transfected with a non-silencing scrambled siRNA.

I examined whether a reduction in N-cad levels in HTR-8/SVneo cells resulted in a concomitant decrease in their invasive capacity. The number of cells that penetrated the Matrigel and reached the underlying side of the filter of the Transwell invasion chambers was significantly lower in cultures of HTR-8/SVneo cells transfected with N-cad siRNA *versus* cell cultures that were transfected with non-silencing scrambled siRNA (Figure 4.12).

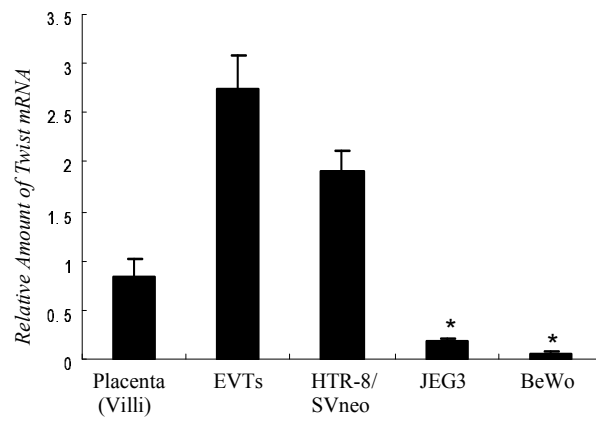
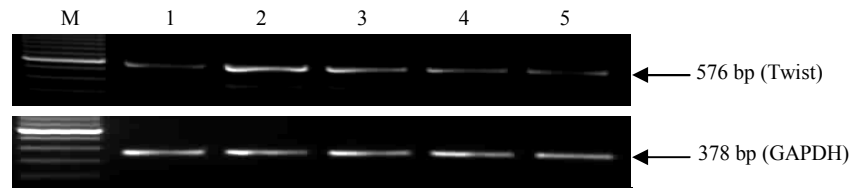
To determine whether the reduction in the invasive capacity of HTR-8/SVneo cells transfected with N-cad siRNA was potentially caused by a loss of N-CAD cell-cell adhesion function, HTR-8/SVneo cells were cultured in the presence of a N-CAD function-perturbing antibody that binds to the N-CAD extracellular domain. The invasive ability of the antibody-treated cells was then assayed as previously described. A pan-cadherin antibody that binds to the N-CAD intracellular domain was used as the control. My results showed that an N-CAD extracellular domain-specific antibody-mediated perturbation of endogenous N-CAD function led to a significantly less invasive

phenotype in HTR-8/SVneo cells, when compared to cells cultured with the control pan-cadherin antibody (Figure 4.13).

Figure 4.1. Twist mRNA and protein levels in human placenta, highly invasive EVT, and poorly invasive trophoblastic cell lines. A) Semi-quantitative RT-PCR analysis of Twist mRNA levels in first trimester placenta, EVT, HTR-8/SVneo cells, JEG-3 or BeWo cells (lanes 1-5 respectively). A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. The photomicrographs were scanned using a laser densitometer. The absorbance values for Twist mRNA were then standardized to the absorbance value obtained for GAPDH mRNA levels. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extract prepared from a first trimester placenta, EVT, HTR-8/SVneo cells, JEG-3 cells or BeWo cells (lanes 1-5 respectively). Western blot analysis was performed using a polyclonal antibody against TWIST. The resultant fluorograms were scanned and the absorbance values obtained for TWIST protein levels were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT control).

Figure 4.1

A



B

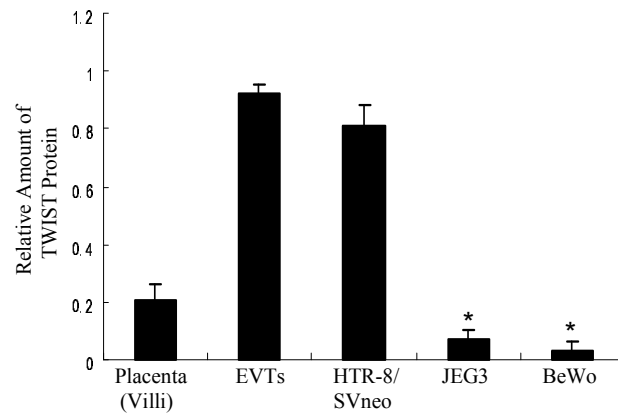
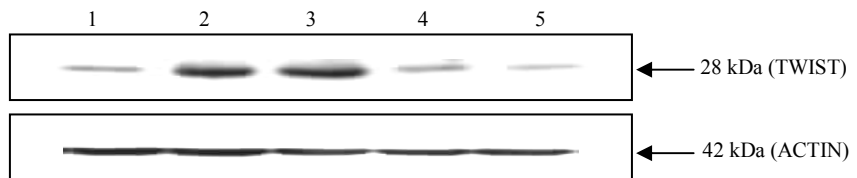
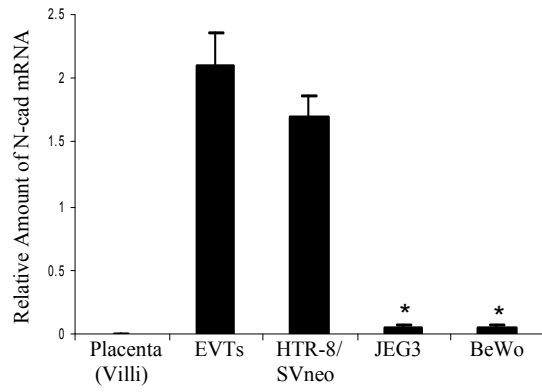
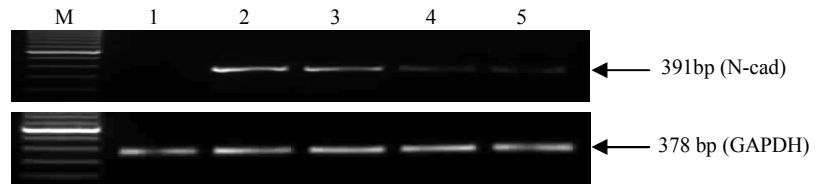




Figure 4.2. N-cad mRNA and protein levels in human placenta, highly invasive EVT<sub>s</sub>, and poorly invasive trophoblastic cell lines. A) Semi-quantitative RT-PCR analysis of N-cad mRNA levels in first trimester placenta, EVT<sub>s</sub>, HTR-8/SVneo cells, JEG-3 or BeWo cells (lanes 1-5 respectively). A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. The photomicrographs were scanned using a laser densitometer. The absorbance values for N-cad mRNA were then standardized to the absorbance value obtained for GAPDH mRNA levels. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracts prepared from first trimester placenta, EVT<sub>s</sub>, HTR-8/SVneo cells, JEG-3 cells or BeWo cells (lanes 1-5 respectively). Western blot analysis was performed using a monoclonal antibody against N-CAD. The resultant fluorograms were scanned and the absorbance values for N-CAD were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT control).

Figure 4.2

A



B

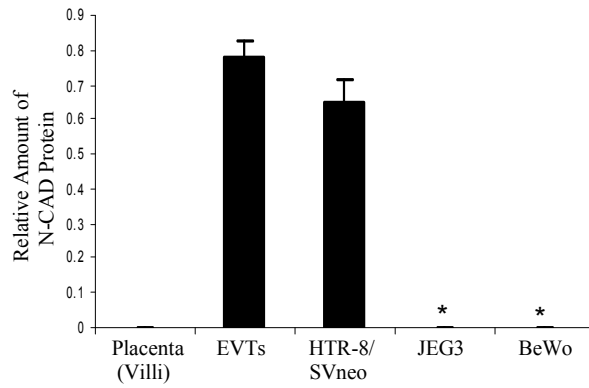
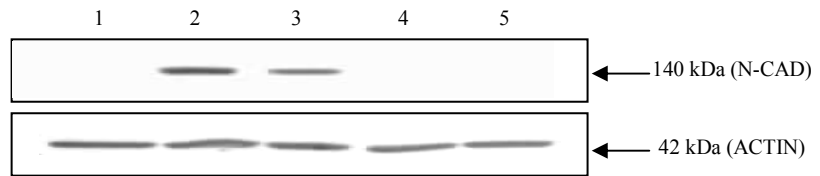


Figure 4.3. Regulatory effects of IL-1 $\beta$  and TGF- $\beta$ 1 on EVT invasion. EVTs were either treated with IL-1 $\beta$  (A) or TGF- $\beta$ 1 (B) for 24 h. The cells were then placed in the upper well of Transwell invasion chambers. After a further 24 h of incubation, the porous membranes from the bottom of the Transwell were removed and fixed, stained, and mounted upside-down on a glass microscope slide. Invasion was determined by counting the number of cells that had invaded through the thin pre-coated layer of Matrigel on the top of the porous (8  $\mu$ m) membrane and migrated through the pores to the underside of the membrane. Cells were visualized using a light microscope, and counted in three randomly selected fields of each membrane. Each cell line was plated in triplicate wells, and the experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to control).

Figure 4.3

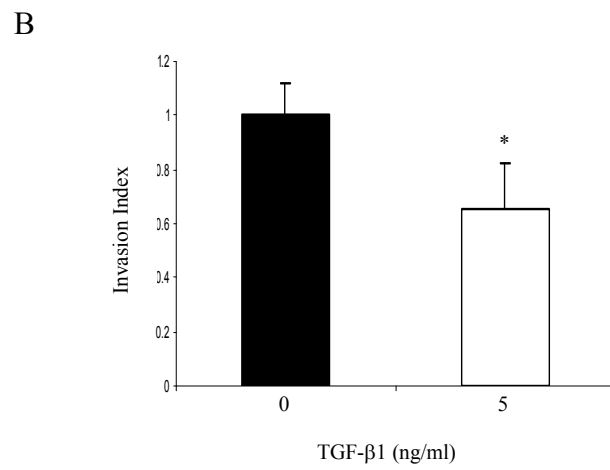
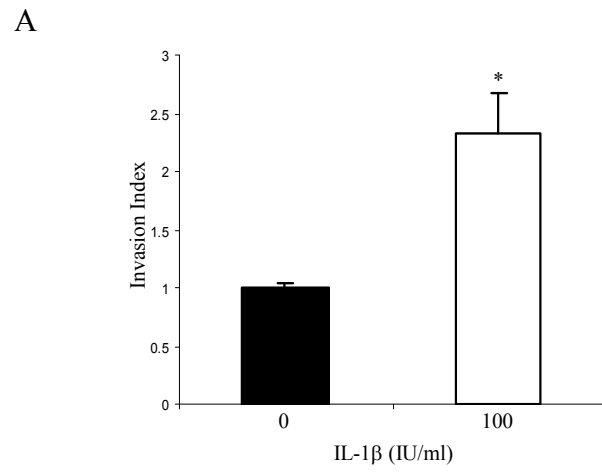
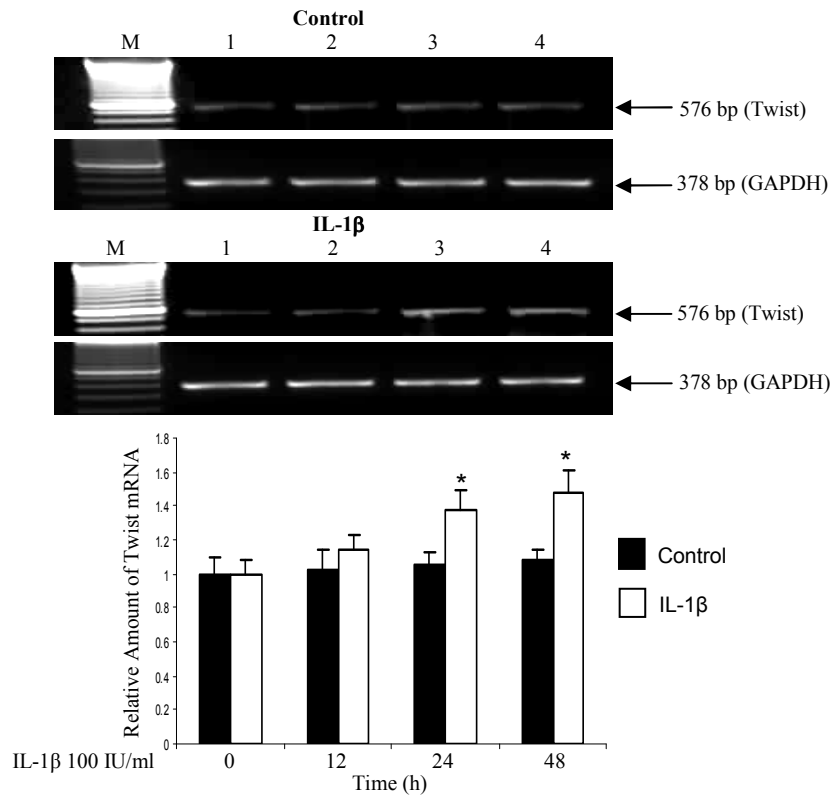


Figure 4.4. Time-dependent effects of IL-1 $\beta$  on Twist mRNA and protein levels in EVT<sub>s</sub>.

A) Semi-quantitative RT-PCR analysis of Twist mRNA levels in EVT<sub>s</sub> cultured in the presence of 100IU/ml IL-1 $\beta$  for 0, 12, 24, or 48 h (lanes 1-4, respectively). A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. A representative photomicrograph of the ethidium bromide-stained gels is presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 h control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT<sub>s</sub> cultures treated with 100IU/ml IL-1 $\beta$  for 0, 12, 24, or 48 h (lanes 1-4, respectively) and probed with a rabbit polyclonal antibody against TWIST or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis and from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 h control).

Figure 4.4

A



B

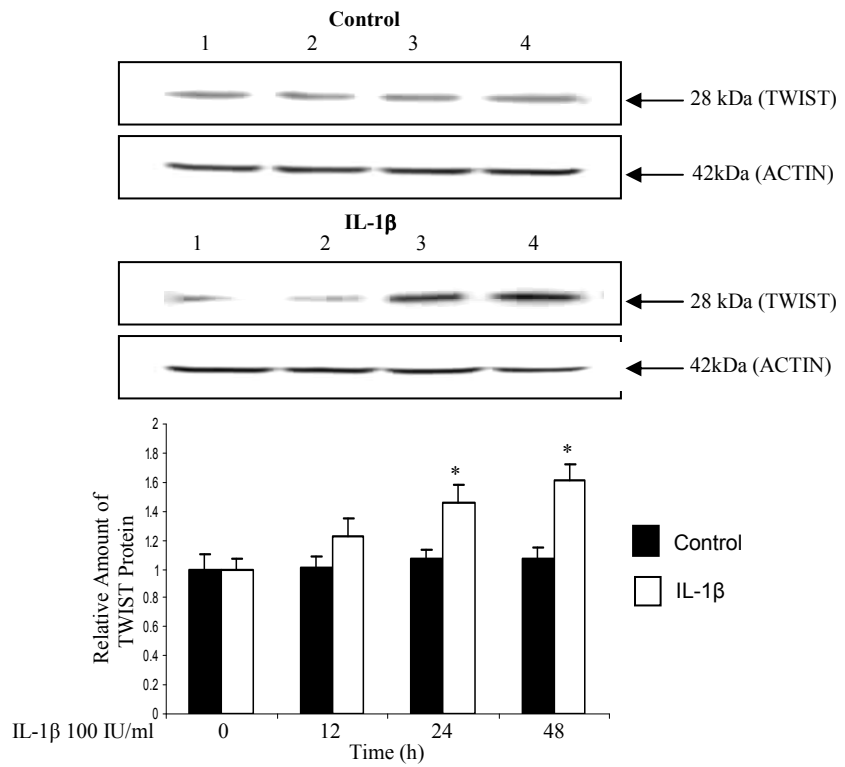
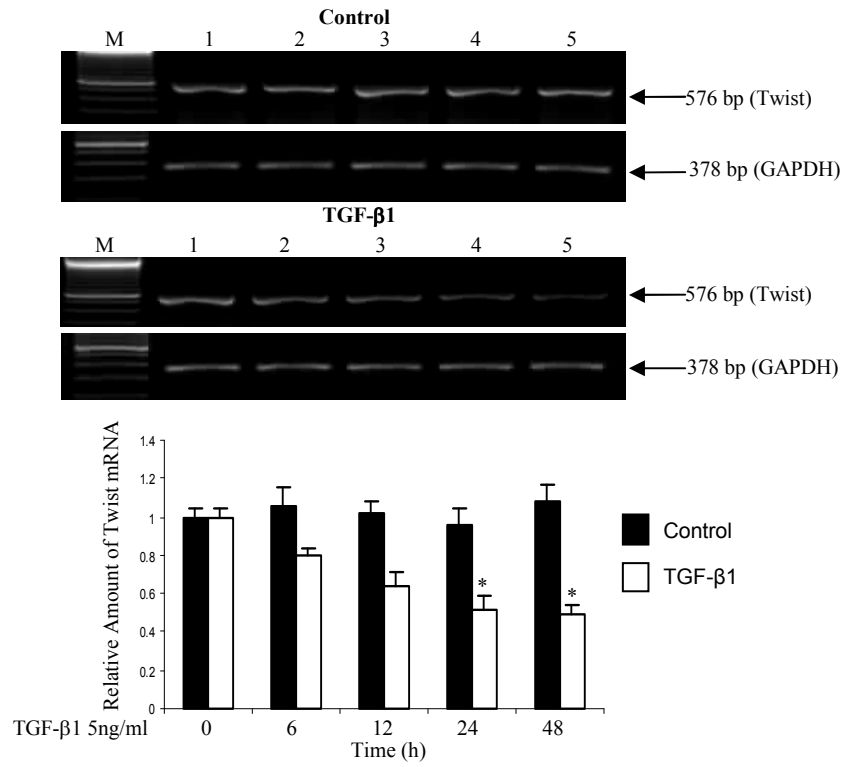


Figure 4.5. Time-dependent effects of TGF- $\beta$ 1 on Twist mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Twist mRNA levels in EVT<sub>s</sub> cultured in the presence of 5 ng/ml TGF- $\beta$ 1 for 0, 6, 12, 24, or 48 h (lanes 1-5, respectively). A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. A representative photomicrograph of the ethidium bromide-stained gels is presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 h control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT<sub>s</sub> cultures treated with 5 ng/ml TGF- $\beta$ 1 for 0, 6, 12, 24, or 48 h (lanes 1-5, respectively) and probed with a rabbit polyclonal antibody against TWIST or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from this analysis and from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 h control).

Figure 4.5

A



B

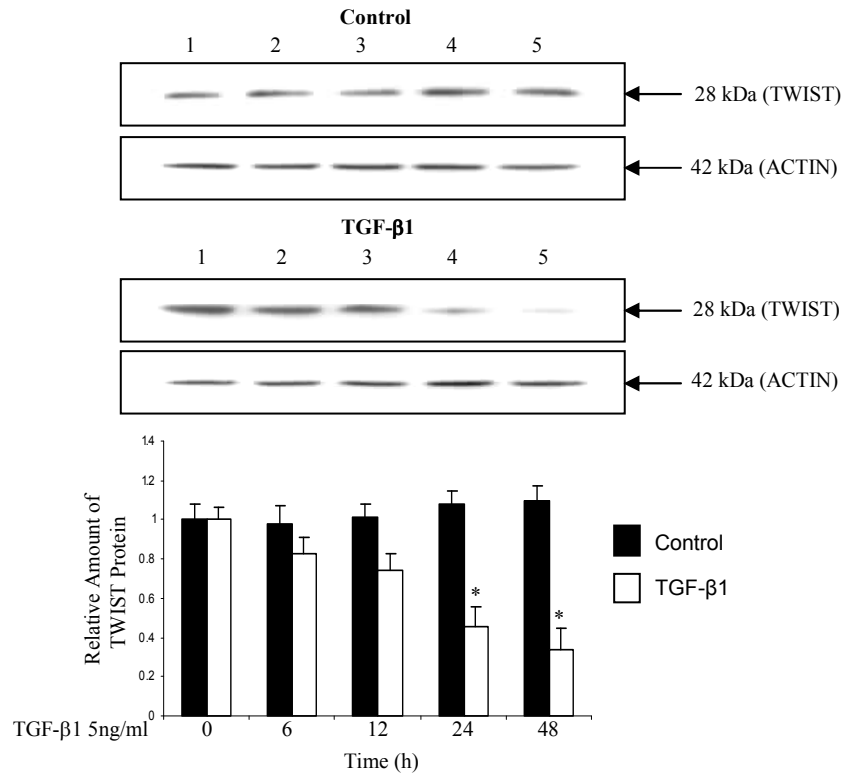
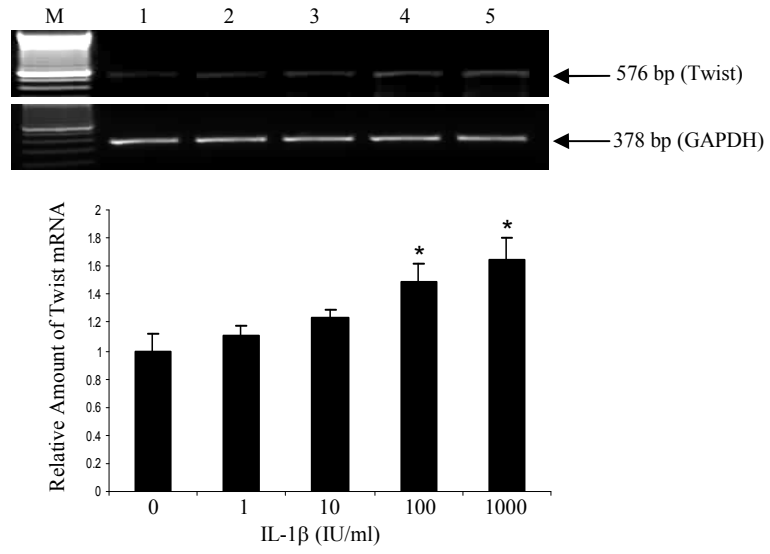




Figure 4.6. Concentration-dependent effects of IL-1 $\beta$  on Twist mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Twist mRNA in EVT<sub>s</sub> cultured in the presence of IL-1 $\beta$  (0, 1, 10, 100 or 1000 IU/ml; lanes 1-5, respectively) for 24 h. A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. Representative photomicrographs of the resultant ethidium bromide-stained gels are presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 IU/ml control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT<sub>s</sub> cultures treated with IL-1 $\beta$  (0, 1, 10, 100 or 1000 IU/ml; lanes 1-5, respectively) for 24 h and probed with rabbit polyclonal antibodies against TWIST or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from this analysis and from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 IU/ml control).

Figure 4.6

A



B

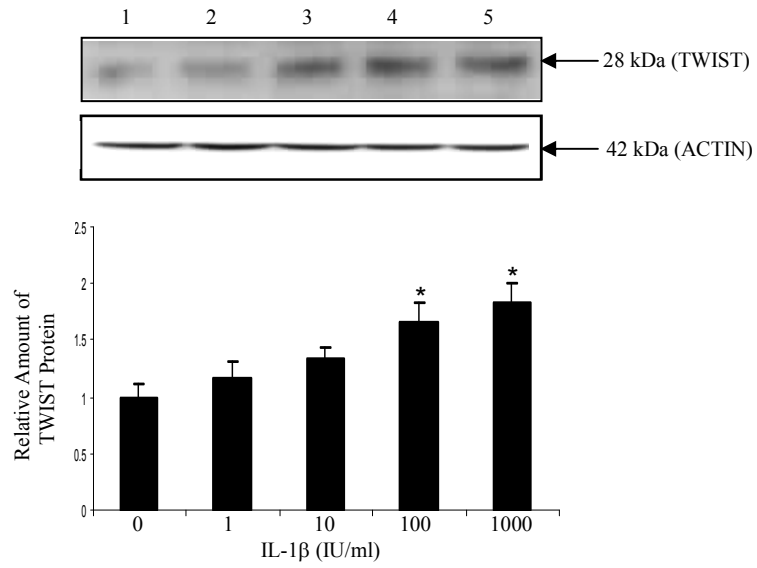
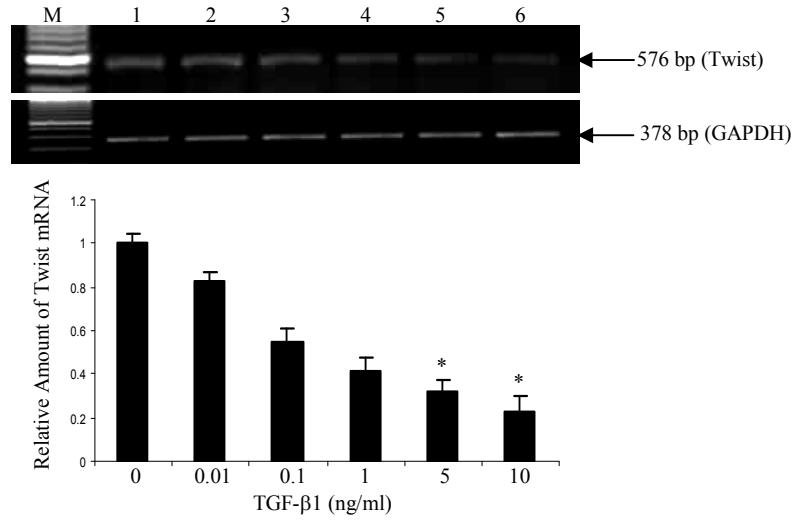


Figure 4.7. Concentration-dependent effects of TGF- $\beta$ 1 on Twist mRNA and protein expression levels in EVT. A) Semi-quantitative RT-PCR analysis of Twist mRNA in EVTs cultured in the presence of TGF- $\beta$ 1 (0, 0.01, 0.1, 1, 5 or 10 ng/ml; lanes 1-6, respectively) for 24 h. A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. Representative photomicrographs of the resultant ethidium bromide-stained gels are presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 ng/ml control) in the graph. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVTs cultures treated with TGF- $\beta$ 1 (0, 0.01, 0.1, 1, 5 or 10 ng/ml; lanes 1-6, respectively) for 24 h and probed with rabbit polyclonal antibodies against TWIST or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from this analysis and from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 ng/ml control).

Figure 4.7

A



B

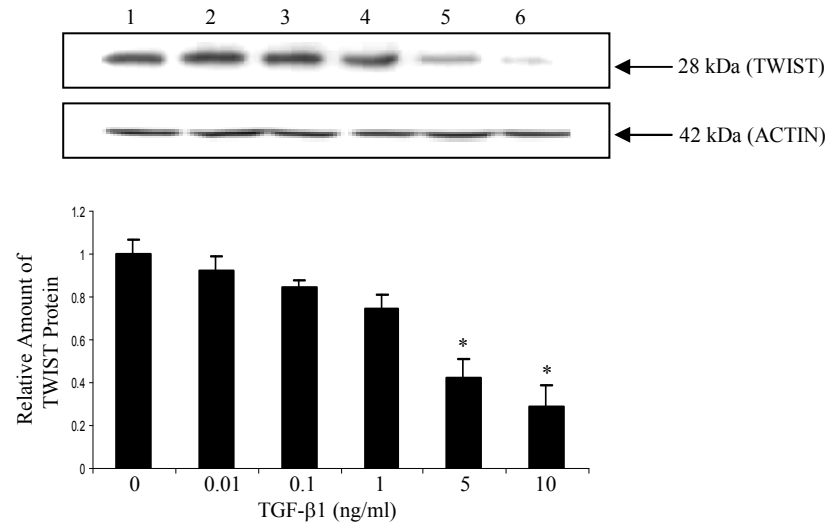
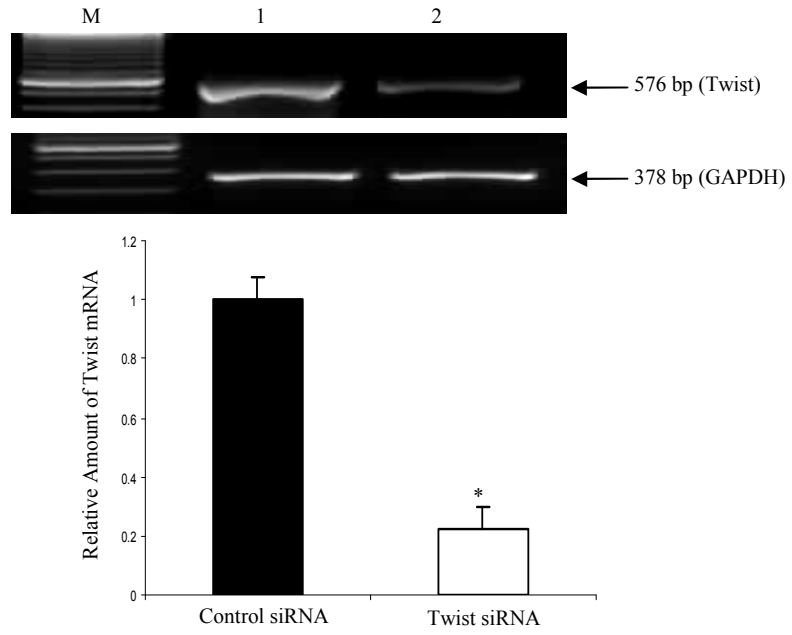


Figure 4.8. Effects of Twist siRNA on Twist mRNA and protein levels in HTR-8/SVneo cell cultures. A) Semi-quantitative RT-PCR analysis of Twist mRNA levels in cells transfected with a scrambled control siRNA (lane 1) or siRNA specific for Twist (lane 2) for 36 h. A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. Values for Twist mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from HTR-8/SVneo cells cultured with a scrambled control siRNA (lane 1) or siRNA specific for Twist (lane 2) for 36 h and probed with rabbit polyclonal antibodies against TWIST or human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for TWIST protein levels were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from this analysis and from three other studies were standardized to the scrambled control siRNA and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA).

Figure 4.8

A



B

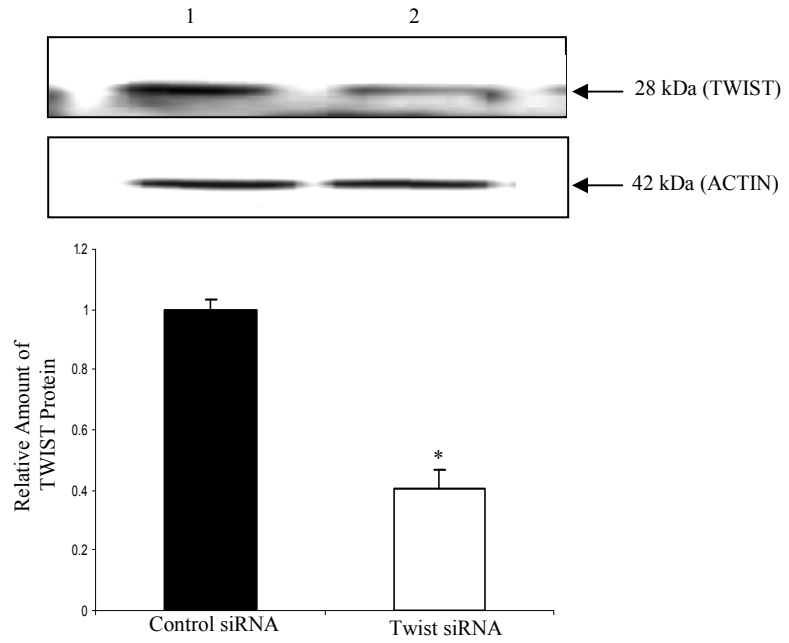
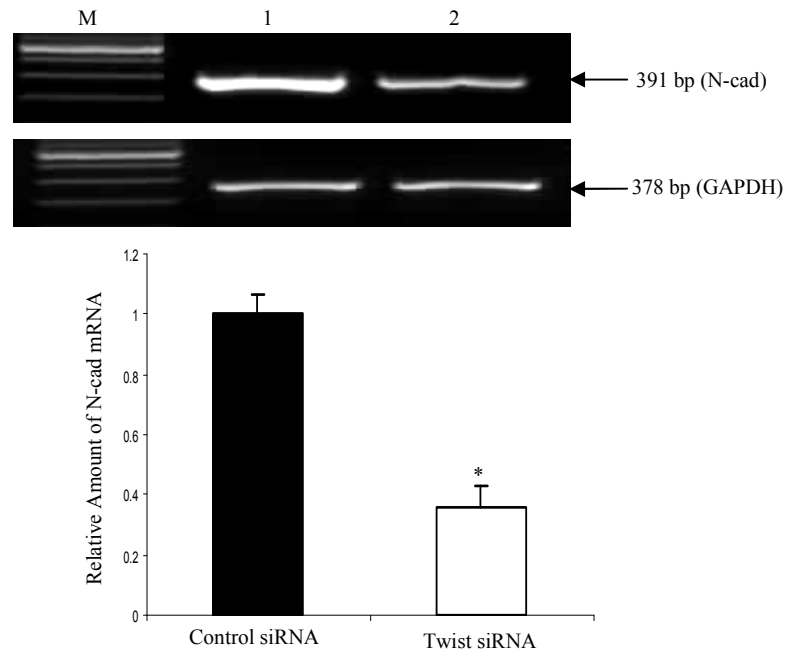


Figure 4.9. Effects of Twist siRNA on N-cad mRNA and protein levels in HTR-8/SVneo cell cultures. A) Semi-quantitative RT-PCR analysis of N-cad mRNA levels in cells transfected with a scrambled control siRNA (lane 1) or siRNA specific for Twist (lane 2) for 36 h. A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. Values for N-cad mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from HTR-8/SVneo cells transfected with a scrambled control siRNA (lane 1) or siRNA specific for TWIST (lane 2) for 36 h and probed with mouse monoclonal antibody against N-CAD or rabbit polyclonal antibody against human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for N-CAD protein levels were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from this analysis and from three other studies were standardized to the scrambled control siRNA and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA).

Figure 4.9

A



B

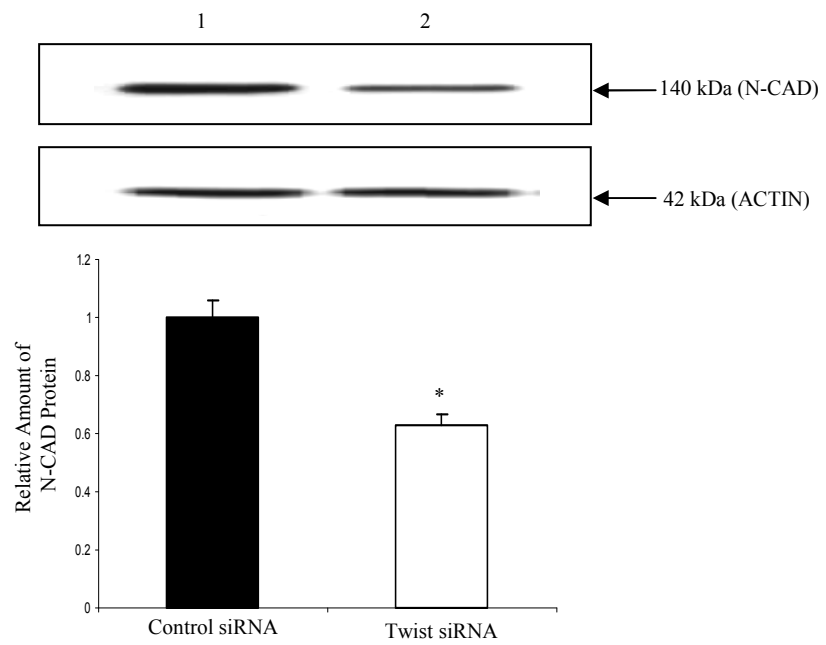




Figure 4.10. Reduced Twist expression decreases the invasive capacity of HTR-8/SVneo cells. HTR-8/SVneo cells were transfected with a scrambled control siRNA (lane 1) or siRNA specific for Twist (lane 2) for 24 h. The cells were then placed in the upper wells of Transwell invasion chambers. After a further 24 h of culture, the porous membranes from the bottom of the Transwells were removed and fixed, stained and mounted upside-down on a glass microscope slide. Invasion was determined by counting the number of cells that had invaded through the thin pre-coated layer of Matrigel on the top of the porous (8  $\mu$ m) membrane and migrated through the pores to the underside of the membrane. Cells were visualized using a light microscope, and counted in three randomly selected fields of each membrane. Each cell line was plated in triplicate wells, and with the experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to HTR-8/SVneo scrambled control siRNA).

Figure 4.10

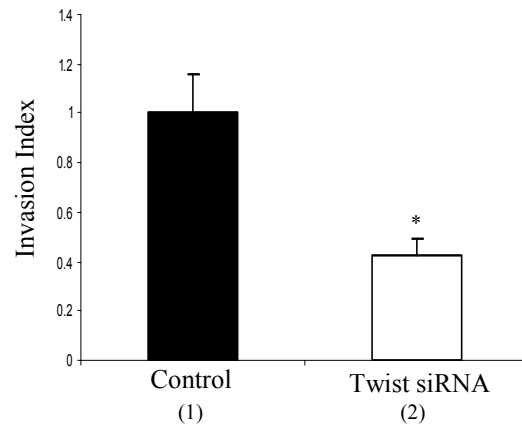
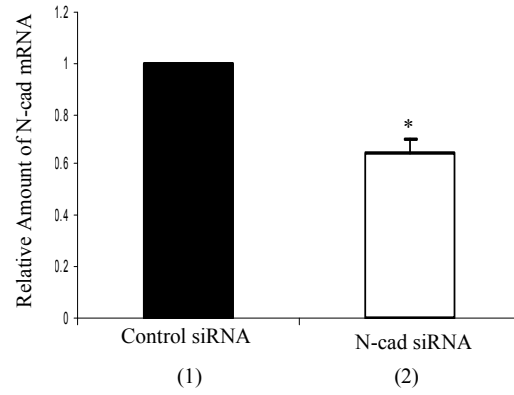


Figure 4.11. Effects of N-cad siRNA on N-cad mRNA and protein levels in HTR-8/SVneo cell cultures. A) Real-time PCR analysis of N-cad mRNA levels transfected with a scrambled control siRNA (lane 1) or siRNA specific for N-cad (lane 2) for 36 h. Values for the levels of the N-cad mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA). B) Representative fluorogram of a Western blot containing total protein extracted from HTR-8/SVneo cells transfected with a scrambled control siRNA (lane 1) or siRNA specific for N-cad (lane 2) for 36 h and probed with mouse monoclonal antibody directed against N-CAD or rabbit polyclonal antibody against human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for N-CAD protein levels were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the corresponding samples. The results derived from both these analyses and from three other sets of experiments were standardized to the scrambled control siRNA and are represented (mean  $\pm$  SEM., n = 4) in the bar graphs (\*,  $P < 0.05$  compared to scrambled control siRNA).

Figure 4.11

A.



B.

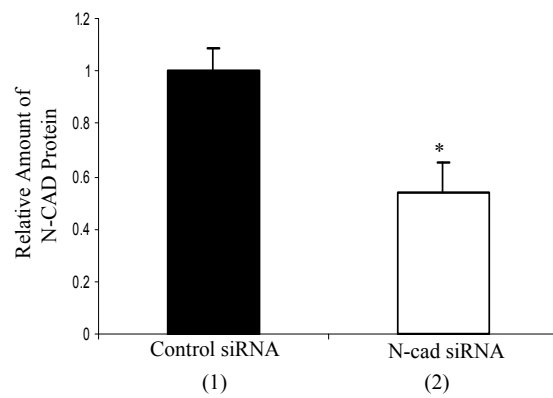
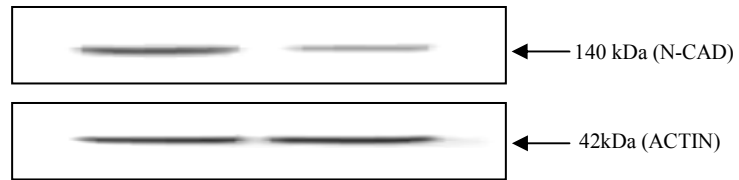


Figure 4.12. Reduced N-cad levels decrease the invasive capacity of HTR-8/SVneo cells. HTR-8/SVneo cells were transfected with a scrambled control siRNA (lane 1) or siRNA specific for N-cad (lane 2) for 24 h. The cells were then placed in the upper wells of Transwell invasion chambers. After a further 24 h of incubation, the porous membranes from the bottom of the Transwells were removed and fixed, stained and mounted upside-down on a glass microscope slide. Invasion was determined by counting the number of cells that had invaded through the thin pre-coated layer of Matrigel on the top of the porous (8  $\mu$ m) membrane and migrated through the pores to the underside of the membrane. Cells were visualized using a light microscope, and were counted in three randomly selected fields of each membrane. Each cell line was plated in triplicate wells, and the experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to scrambled control siRNA).

Figure 4.12

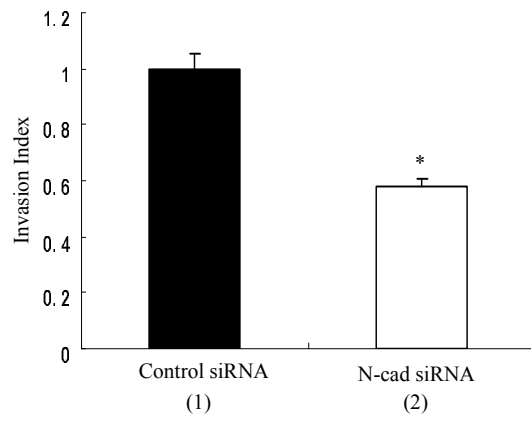
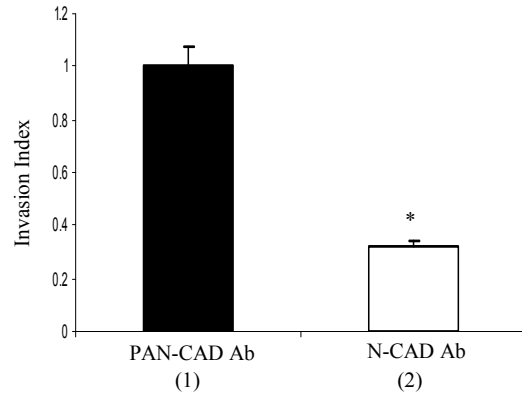


Figure 4.13. Disruption of N-CAD function decreases the invasive ability of HTR-8/SVneo cells. HTR-8/SVneo cells were treated with a control pan-cadherin antibody (lane 1) or an N-CAD function-perturbing antibody (lane 2) for 24 h. The cells were then placed in the upper wells of Transwell invasion chambers. After a further 24 h of incubation, the porous membranes from the bottom of the Transwells were removed and fixed, stained, and mounted upside-down on a glass microscope slide. Invasion was determined by counting the number of cells that had invaded through the thin pre-coated layer of Matrigel on the top of the porous (8  $\mu$ m) membrane and migrated through the pores to the underside of the membrane. Cells were visualized using a light microscope, and were counted in three randomly selected fields of each membrane. Each cell line was plated in triplicate wells, and the experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to control pan-cadherin (PAN-CAD) antibody).

Figure 4.13





### 4.3: Discussion and summary

My studies demonstrate that Twist and N-cad are highly expressed in highly invasive EVT<sub>s</sub> propagated from first trimester human placenta and the HTR-8 EVT cell line but are not readily detectable in the poorly invasive JEG-3 and BeWo cell lines. In addition, IL-1 $\beta$  and TGF- $\beta$ 1 were found to have differential effects on Twist mRNA and protein levels in primary cultures of EVT<sub>s</sub>, and this suggests that these molecules play important roles in human trophoblastic cell invasion.

Extravillous trophoblasts (EVT<sub>s</sub>) can be divided into two populations: 1) interstitial cytotrophoblasts that will invade into the decidual stroma and superficial myometrium; and 2) endovascular cytotrophoblasts which will invade into the lumen of the spiral arteries (Pijnenborg *et al.*, 1981; Pijnenborg 1983; Roberston *et al.*, 1986). In order for the human placenta to properly form, the trophoblast must invade into the uterus, which involves attachment of these cells to the extracellular matrix (ECM), degradation of the matrix, and migration. The spiral arteries of the placental bed also have to undergo a certain degree of alteration. The interaction between the invasive cytotrophoblast and the spiral artery vessel wall is the major step in achieving these physiological modifications (Lyll, 2006). Trophoblast invasion is controlled by cell adhesion molecules including cadherins, which are expressed on the surface of cytotrophoblasts that interact with the ECM of the decidua (Kreis *et al.*, 1993; Alberts *et al.*, 1994).

The cadherin N-CAD has been shown to have the ability to mediate homotypic cell aggregation as well as the ability to form heterotypic adhesions in various cell types including stromal fibroblasts, vascular endothelial cells, smooth muscle cells, and

myofibroblasts (Hazan *et al.*, 1997; Tran *et al.*, 1999; Li *et al.*, 2001; De Wever *et al.*, 2003). For instance, by increasing the interaction with the surrounding stroma, N-CAD is able to promote invasion and metastasis (Hazan *et al.*, 1997). In addition, the transcription factor Twist has been reported to increase vascular volume and vascular permeability by increasing vascular endothelial growth factor (VEGF) synthesis, and inducing *in vivo* angiogenesis (Mironchik *et al.*, 2005).

In my studies, when I used a siRNA strategy targeting Twist in human trophoblastic cells, the invasive ability of these cells was significantly reduced. Others have also reported that inactivation of Twist suppressed the migration and invasion abilities of androgen-independent prostate cancer cells (Kwok *et al.*, 2005). In my studies, when the invasive ability of trophoblastic cells was reduced in the presence of Twist siRNA, I also observed decreased N-cad mRNA and protein levels in these cells. This suggests that Twist regulates human trophoblast invasion *via* N-cad. Furthermore, my results show that by directly limiting N-cad expression in a Twist-independent manner (i.e. with N-cad-directed siRNA) in human trophoblastic cells, the invasive ability of these cells was significantly reduced.

My loss-of-function studies in which either Twist or N-cad was targeted have clearly shown a concomitant reduction in the invasive ability of human trophoblastic cells. Furthermore, by blocking the extracellular domain of N-CAD with a function-perturbing antibody, the invasive ability of these trophoblastic cells was reduced. This novel finding further strengthens the growing consensus that trophoblast invasion utilizes similar molecular mechanisms to those of tumour cell invasion.

As previously mentioned, decreased Twist expression, which is involved in cancer metastasis, was found to be associated with down-regulation of N-cad in a variety of cancer tumours such as osteosarcomas (Guo *et al.*, 2007). However, in my study, I could not determine whether TWIST interacts with N-cad directly or indirectly. In a prostate cell line, TWIST did not show an increase in promoter-binding activity, but was found to regulate N-cad expression through its direct interaction with an E-box regulatory element located within the first intron of the N-cad gene (Alexander *et al.*, 2006).

Rosivatz *et al.* (2004) suggest that E-cad transcriptional repressors may not play a major role in colon cancer pathogenesis, and other studies have not always found a correlation between reduced E-CAD immunohistochemistry and tumour progression. It seems that N-cad induced invasion activities can even overcome the E-cad tumour suppressive function (Hazan *et al.*, 1997; Nieman *et al.*, 1999). In my preliminary data, I observed that when I silenced Twist expression in human trophoblastic cells, E-cad expression levels remain unchanged (data not shown). This requires further validation, but indicates that the “cadherin switch” varies in a tumour or tissue-specific manner.

Collectively, my results have clearly identified TWIST and its associated protein, N-CAD, to be key molecules in human EVT invasion (Figure 3.14). To my knowledge, this is the first study to demonstrate the regulation of trophoblast invasion by Twist through its role in the induction of N-cad gene expression. TWIST and N-CAD may serve as useful diagnostic and prognostic tools or novel therapeutic targets for human trophoblastic diseases such as miscarriage, intrauterine growth restriction (IUGR), or preeclampsia.

## Extravillous pathway (invasion)

(Twist  $\longrightarrow$  N-cad  $\longrightarrow$  Invasion)

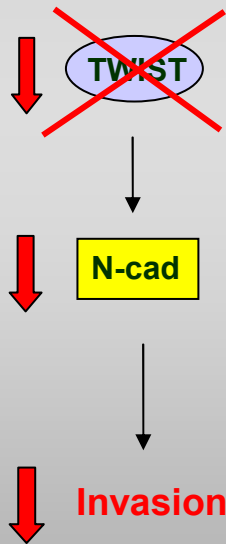


Figure 4.14. A schematic diagram of a proposed role of Twist in regulating N-cad-mediated differentiation of human trophoblastic cells. Silencing Twist expressing by siRNA strategy reduces N-cad expression level and reduces the invasion ability of human trophoblastic cells.

## **CHAPTER 5: A KEY ROLE FOR RUNX2 IN HUMAN TROPHOBLAST INVASION.**

### **5.1: Introduction and rationale**

The trophoblastic cells form the outer layer of the blastocyst and play an essential role in implantation and placentation during human pregnancy. The cytotrophoblast stem cells are specialized epithelial cells of the placenta which can undergo two differentiation pathways (Zhou *et al.*, 1997). In one pathway, the cytotrophoblast cells fuse to form multinucleated syncytial trophoblast cells, which are involved in maternal-fetal exchanges and placental endocrine functions. In the other pathway, the cytotrophoblasts differentiate into the invasive extravillous cytotrophoblast (EVT) cells. These EVT cells invade the maternal uterine wall and its blood vessels to establish the flow of oxygenated blood to the placenta (Aplin *et al.*, 1991). Proper trophoblast invasion is critical for a healthy pregnancy, and insufficient invasion is associated with preeclampsia, intrauterine growth restriction and recurrent miscarriage (Goldman-Wohl and Yagel, 2002).

Cells at the maternal-fetal interface are exposed to numerous cytokines, growth factors and hormones that play critical roles in mediating the processes required for cell invasion (Chakraborty *et al.*, 2002). The processes required for trophoblast invasion include degradation and remodelling of the extracellular matrix (ECM) components, and regulated changes in cell-cell and cell-matrix interactions (MacCalman *et al.*, 1998, Chakraborty *et al.*, 2002; Cohen *et al.*, 2006).

The RUNX proteins (runt-related transcription factor) are a family of transcription factors that contain a DNA-binding runt domain (Ito, 1999). The Runx2 gene (also

known as PEBP2 $\alpha$ /AML3/CBFA1) is essential for osteoblast development and proper bone formation (Otto *et al.*, 1997). In cancer cells, Runx2 is capable of activating the expression of adhesion proteins, matrix metalloproteinases (MMPs) and angiogenic factors known to be associated with invasive properties of metastatic cancer cells (Pratap *et al.*, 2006).

Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a cytokine that plays a major regulatory role in the establishment of pregnancy (Salamonsen *et al.*, 2000, 2003; Fazleabas *et al.*, 2004). In particular, IL-1 $\beta$  has been shown to increase the invasiveness of primary cultures of trophoblastic cells (Librach *et al.*, 1994; Simon *et al.*, 1994; Karmakar and Das, 2002). Conversely, another cytokine, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is highly expressed in both fetal and maternal cellular compartments of the term human placenta and reduces trophoblastic cell invasion (Lala and Graham, 1990; Graham and Lala, 1991, 1992; Godkin and Dore, 1998), TGF- $\beta$ 1 also plays a key regulatory role in placenta development and function (Graham and Lala, 1991). Nevertheless, the precise mechanisms that regulate trophoblast invasion are not fully understood.

Cadherins play an important role in embryogenesis. The neural cell adhesion molecule, N-cadherin (N-CAD), serves as a key molecule during gastrulation and neural crest development. In previous studies, I have identified that N-cad plays a role in human trophoblastic cell invasion. Furthermore, in cancer, the expression of N-cad in epithelial cells alters cell morphology to a fibroblastic phenotype, enhancing their motility and invasive potential (Derycke and Bracke, 2004). Studies have shown that N-cad plays critical roles in the invasive properties of various cancer cell types, such as those of the

colon, breast and pancreas (Nieman *et al.*, 1999; Nakajima *et al.*, 2003; Rieger-Christ *et al.*, 2004).

In view of the above observations, I hypothesized that Runx2 expression plays a key role in regulating human trophoblastic cell invasion by modulating N-cad expression. In this study, by using semi-quantitative RT-PCR and Western blotting, I have examined Runx2 mRNA and protein levels in human placental tissues and cells. Furthermore, I have examined the ability of IL-1 $\beta$  and TGF- $\beta$ 1 to regulate Runx2 mRNA and protein expression levels in primary cultures of EVT<sub>s</sub>. By using the Matrigel invasion assay, I have also identified a role for Runx2 in human trophoblastic cell invasion through silencing Runx2 expression using a siRNA strategy. Finally, I determined whether N-cad can be regulated by Runx-2 in human trophoblastic cell invasion.

## **5.2: Results**

### **5.2.1: Runx2 is expressed in human placenta, highly invasive EVT, and poorly invasive trophoblastic cell lines.**

Prior to these studies, nothing was known about the expression of Runx2 in the human placenta. Semi-quantitative RT-PCR and Western blot experiments revealed the presence of Runx2 mRNA and protein in first trimester human placenta. Runx2 mRNA and protein were most abundant in primary EVT and HTR-8/SVneo EVT cells but were present in very low amounts in JEG-3 and BeWo cells (Figure 5.1A and B).

### **5.2.2: Time-dependent effects of IL-1 $\beta$ on Runx2 mRNA and protein levels in human EVT**

IL-1 $\beta$  (100 IU/ml) treatment resulted in a significant increase in Runx2 mRNA levels in primary EVT after 24 h of culture, with maximum levels being detected in cells cultured in the presence of this cytokine for 48 h (Figure 5.2A). Treatment with 100 IU/ml IL-1 $\beta$  for 24 h induced an approximately 1.4-fold increase in Runx2 mRNA, while the same treatment for 48 h induced approximately a 1.5-fold increase. In correspondence with the fold-increases in mRNA, RUNX2 protein levels in EVT cultures increased approximately 1.6-fold by treatment with 100 IU/ml IL-1 $\beta$  for 24 h, and increased approximately 1.8-fold upon treatment for 48 h (Figure 5.2B).



### **5.2.3: Time-dependent effects of TGF- $\beta$ 1 on Runx2 mRNA and protein levels in human EVT<sub>s</sub>**

A significant decrease in Runx2 mRNA levels was detected in EVT<sub>s</sub> cultured in the presence of 5 ng/ml TGF- $\beta$ 1 for 24 h and 48 h (Figure 5.3A). Treatment with 5 ng/ml TGF- $\beta$ 1 for 24 h induced approximately a 30% decrease in Runx2 mRNA, while the same treatment for 48 h induced an approximately 35% decrease in these primary cell cultures. In agreement with the results obtained using semi-quantitative RT-PCR, RUNX2 protein levels decreased approximately 30% upon treatment with 5 ng/ml TGF- $\beta$ 1 for 24 h, and approximately 35% after the same treatment for 48 h (Figure 5.3B).

### **5.2.4: Concentration-dependent effects of IL-1 $\beta$ on Runx2 mRNA and protein levels in human EVT<sub>s</sub>**

Increasing concentrations of IL-1 $\beta$  increased the Runx2 mRNA levels present in primary cultures of human EVT<sub>s</sub> in a concentration-dependent manner (Figure 5.4A). However, significant increases in Runx2 mRNA levels were only observed in EVT<sub>s</sub> treated with higher concentrations of IL-1 $\beta$  (10, 100 or 1000 IU/ml) in these studies. IL-1 $\beta$  at 10 IU/ml induced approximately a 1.3-fold increase in Runx2 mRNA levels, while 100 and 1000 IU/ml induced approximately 1.5 and 1.7-fold increases, respectively.

In agreement with the results obtained using semi-quantitative RT-PCR, IL-1 $\beta$  increased RUNX2 protein levels in EVT cultures in a concentration-dependent manner (Figure 5.4B). In correspondence with the fold-increases in mRNA, RUNX2 protein

levels were increased 1.4-fold by 10 IU/ml of IL-1 $\beta$ , 1.6-fold by 100 IU/ml of IL-1 $\beta$  and 1.8-fold by 1000 IU/ml of IL-1 $\beta$ .

#### **5.2.5: Concentration-dependent effects of TGF- $\beta$ 1 on Runx2 mRNA and protein levels in human EVT**

TGF- $\beta$ 1 decreased Runx2 mRNA levels in primary EVTs in a concentration-dependent manner. A significant decrease in Runx2 mRNA was observed only in EVTs treated with the highest concentrations of TGF- $\beta$ 1 (1 or 10 ng/ml) (Figure 5.5A). TGF- $\beta$ 1 at 1 ng/ml induced a 20% decrease in Runx2 mRNA, while treatment with 10 ng/ml TGF- $\beta$ 1 resulted in a 30% decrease in Runx2 mRNA. TGF- $\beta$ 1 treatment also reduced RUNX2 protein levels in primary cultures of EVTs in a concentration-dependent manner (Figure 5.5B). In accordance with the fold-decreases in mRNA, RUNX2 protein levels were decreased 25% by 5 or 10 ng/ml of TGF- $\beta$ 1.

#### **5.2.6: Attenuation of cytokine-modulated Runx2 mRNA and protein levels in EVTs using neutralizing antibodies directed against IL-1 $\beta$ or TGF- $\beta$ 1**

Function-perturbing monoclonal antibodies directed against either IL-1 $\beta$  or TGF- $\beta$ 1 had no significant effect on Runx2 mRNA and protein levels in my primary EVTs after 24 h of culture (data not shown). However, IL-1 $\beta$ -mediated increases in the Runx2 mRNA and protein levels in these primary cell cultures were inhibited by the addition of an anti-IL-1 $\beta$  neutralizing antibody to the culture medium for 24 h. IL-1 $\beta$  at 100 IU/ml together with anti-IL-1 $\beta$  antibody at 1  $\mu$ g/ml induced approximately a 26% decrease in

Runx2 mRNA compared to the control treatment (IL-1 $\beta$  at 100IU/ml), while IL-1 $\beta$  at 100 IU/ml together with anti-IL-1 $\beta$  antibody at 2  $\mu$ g/ml induced approximately a 35% decrease in Runx2 mRNA compared to the control treatment (IL-1 $\beta$  at 100IU/ml) (Figure 5.6A). In accordance with these fold-decreases in mRNA, RUNX2 protein expression was decreased 30% by 100 IU/ml of IL-1 $\beta$  together with anti-IL-1 $\beta$  antibody at 1  $\mu$ g/ml, and 35% by 100 IU/ml of IL-1 $\beta$  together with anti-IL-1 $\beta$  antibody at 2  $\mu$ g/ml (Figure 5.6B). Similarly, the monoclonal antibody against TGF- $\beta$ 1 abolished the decrease in Runx2 mRNA and protein levels observed in EVT $s$  cultured in the presence of this cytokine. TGF- $\beta$ 1 at 10 ng/ml together with anti-TGF- $\beta$ 1 antibody at 10  $\mu$ g/ml induced approximately a 1.3-fold increase in Runx2 mRNA compared to control (TGF- $\beta$ 1 at 10 ng/ml) (Figure 5.7A). A similar fold-increase (1.4-fold) in RUNX2 protein expression was observed under these conditions (Figure 5.7B).

#### **5.2.7: Inhibition of Runx2 expression down-regulates N-cad mRNA and protein levels and reduces the invasive capacity of HTR-8/SVneo cells**

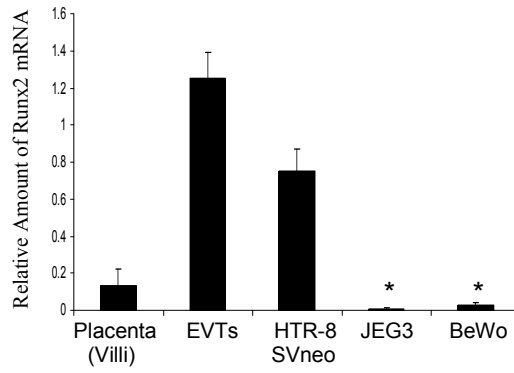
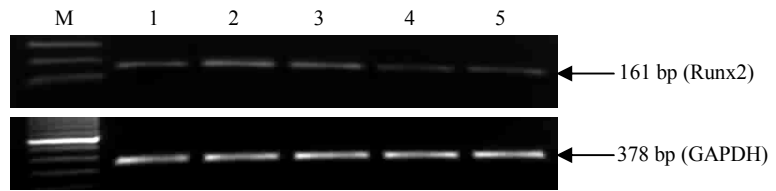
In order to repress Runx2 expression in cultures of HTR-8/SVneo cells, I utilized siRNA complementary to human Runx2 mRNA. Transfection of HTR-8/SVneo cells with this siRNA significantly decreased Runx2 mRNA and protein levels in these cell cultures after 36 h compared to cells transfected with a non-silencing, scrambled siRNA (Figure 5.8A and B). The N-cad mRNA and protein levels were significantly decreased after 36 h of siRNA targeting Runx2, compared to HTR-8/SVneo cells transfected with non-silencing, scrambled siRNA in these studies (Figure 5.9A and B).

I also examined whether a reduction in Runx2 expression in HTR-8/SVneo cells results in a concomitant decrease in their invasive capacity using Matrigel coated Transwell chambers. The number of cells that penetrated the Matrigel and reached the underside of the membrane in the Transwell invasion chambers was significantly lower in cultures of HTR-8/SVneo cells which had been transfected with Runx2 siRNA compared to cell cultures that had been transfected with non-silencing, scrambled siRNA (Figure 5.10).

Figure 5.1. Runx2 mRNA and protein expression levels in human placenta, highly invasive EVT<sub>s</sub> and poorly invasive trophoblastic cell lines. A) Semi-quantitative PCR analysis of Runx2 mRNA levels in first trimester placenta, primary EVT cell cultures, HTR-8/SVneo cells, JEG-3 or BeWo cells (lanes 1-5, respectively). A 100 bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. The photomicrographs were scanned using a laser densitometer. The absorbance values obtained for Runx2 were then standardized to the absorbance value obtained for GAPDH mRNA levels. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT<sub>s</sub> control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extract prepared from first trimester placenta, primary EVT cell cultures, HTR-8/SVneo cells, JEG-3 cells or BeWo cells. Western blot analysis was performed using a polyclonal antibody against RUNX2. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 protein levels were normalized to the absorbance values obtained for  $\beta$ -actin (ACTIN) in the corresponding samples. The experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to EVT control).

Figure 5.1

A



B

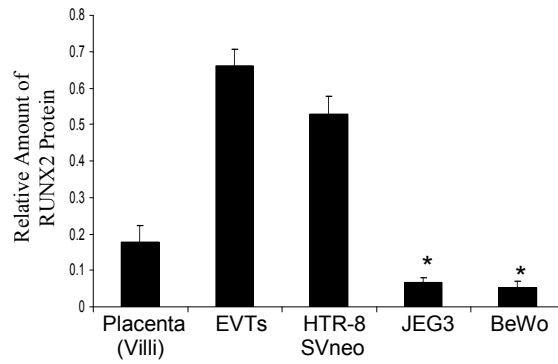
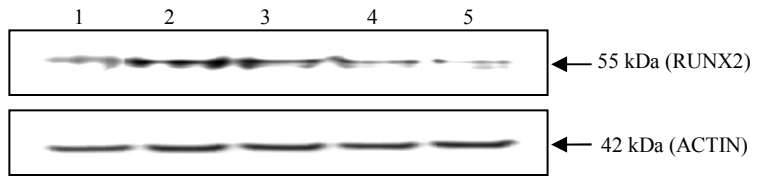
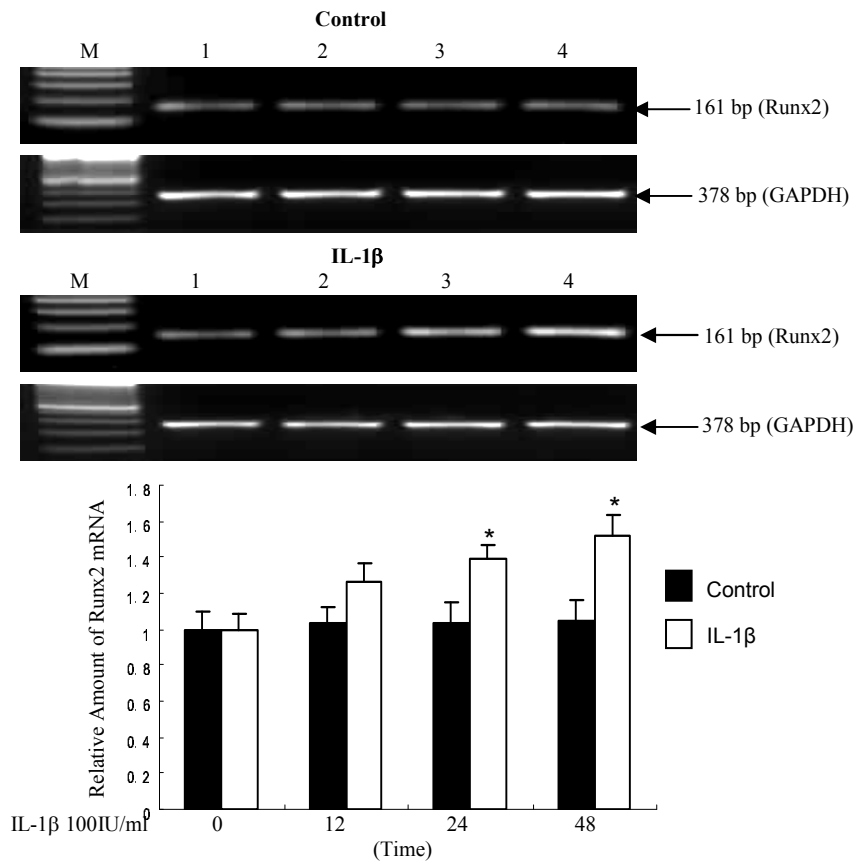


Figure 5.2. Time-dependent effects of IL-1 $\beta$  on Runx2 mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Runx2 mRNA levels in EVT<sub>s</sub> cultured in the presence of 100 IU/ml IL-1 $\beta$  for 0, 12, 24, or 48h (lanes 1-4, respectively). A 100bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. A representative photomicrograph of the ethidium-stained gels is presented. Gels generated from three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0h control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT<sub>s</sub> cultures treated with 100 IU/ml IL-1 $\beta$  for 0, 12, 24, or 48h (lanes 1-4, respectively) and probed with a rabbit polyclonal antibody against RUNX2 or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis and from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0h control).

Figure 5.2

A



B

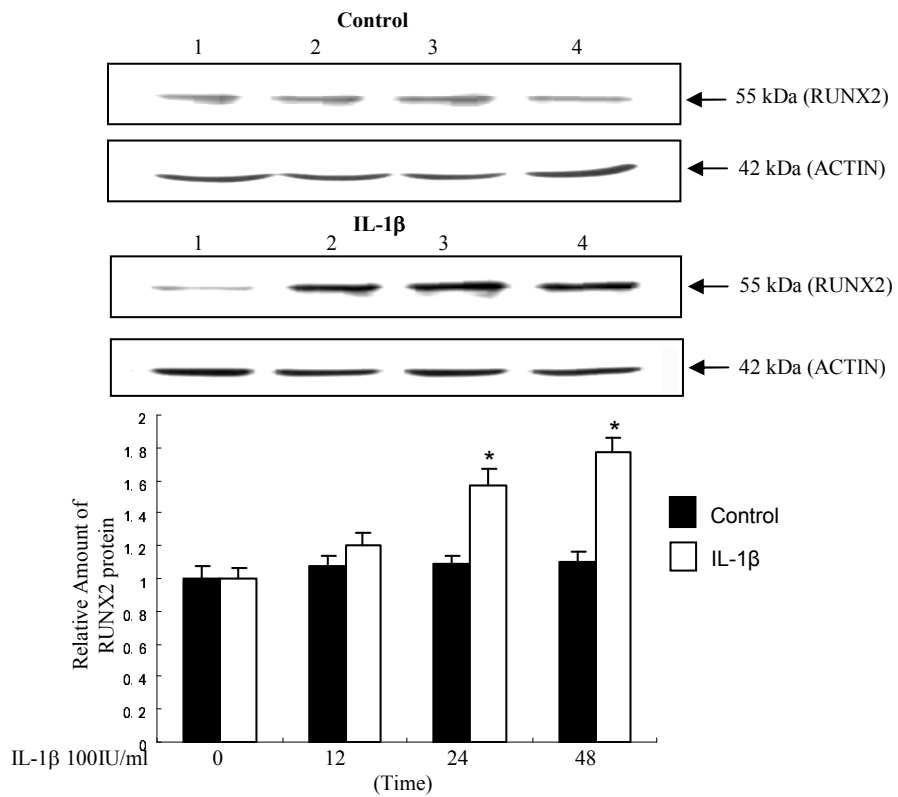
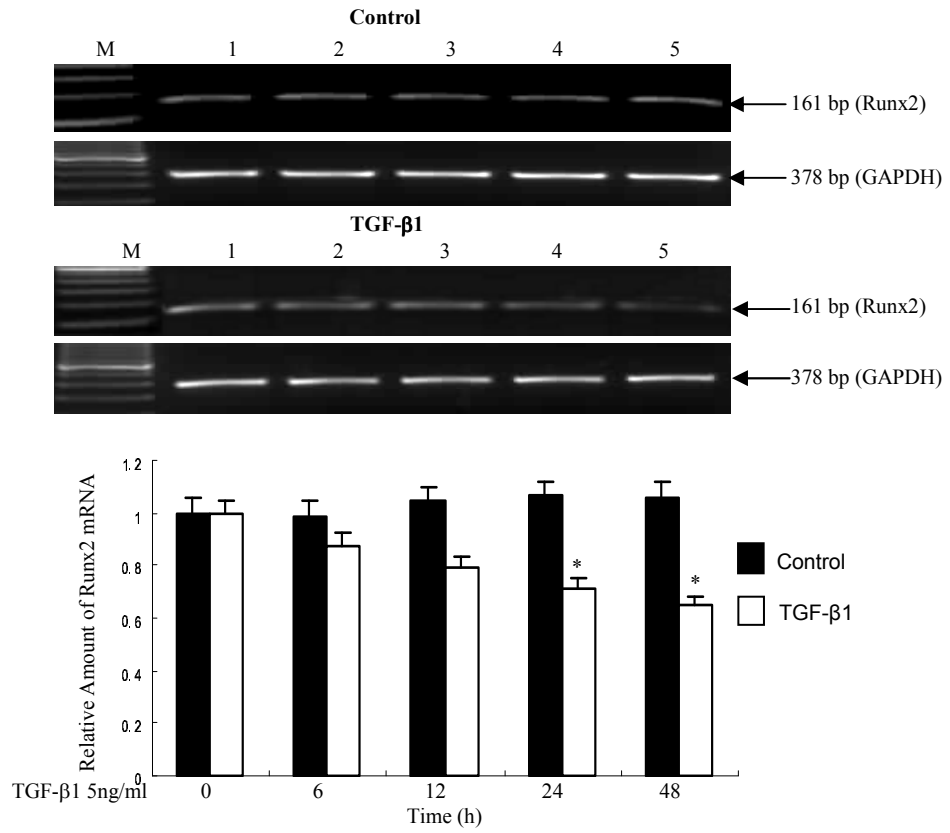




Figure 5.3. Time-dependent effects of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) on Runx2 mRNA and protein levels in EVT. A) RT-PCR analysis of Runx2 mRNA levels in EVT cultured in the presence of 5 ng/ml TGF- $\beta$ 1 for 0, 6, 12, 24 or 48h (lanes 1-5, respectively). A 100-bp ladder is shown in lane M (marker) with the size of the target cDNA indicated at the right. A representative photomicrograph of the resultant ethidium bromide-stained gels is presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are presented as (mean absorbance  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 h control). B) Representative fluorogram of a Western blot containing 30  $\mu$ l of total protein extracted from corresponding EVT cultures treated with 5 ng/ml TGF- $\beta$ 1 for 0, 6, 12, 24 or 48h (lanes 1-5, respectively) and probed with a rabbit polyclonal antibody against RUNX2 or human  $\beta$ -actin. The Amersham enhanced chemiluminescence system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis as well as those from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0h control).

Figure 5.3

A



B

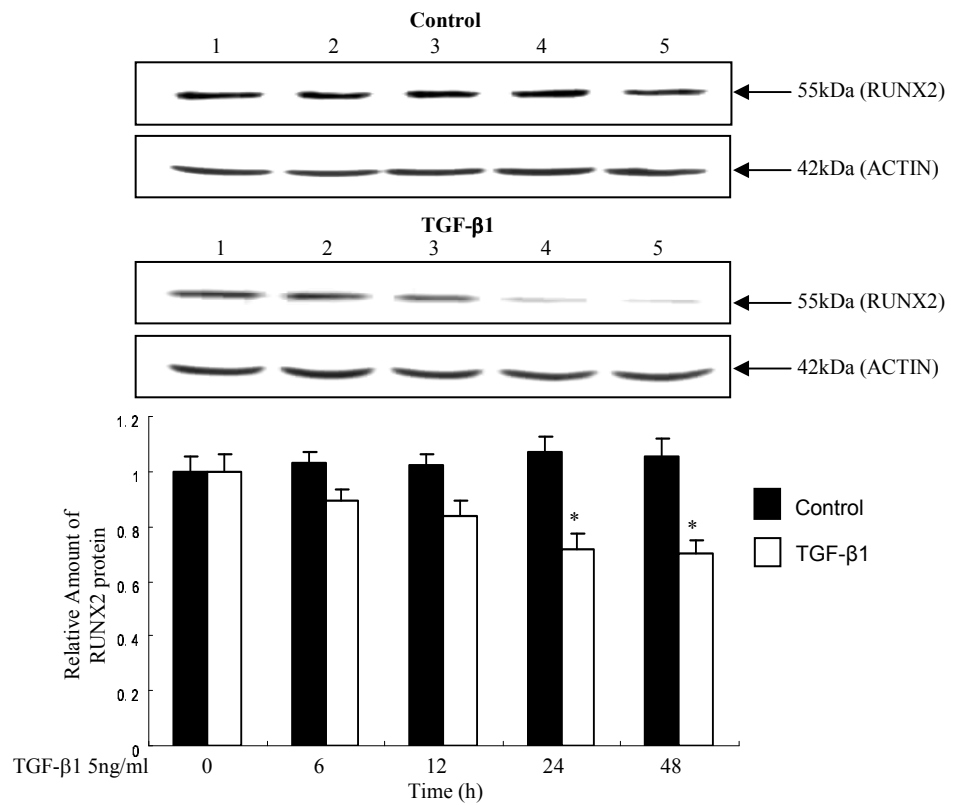
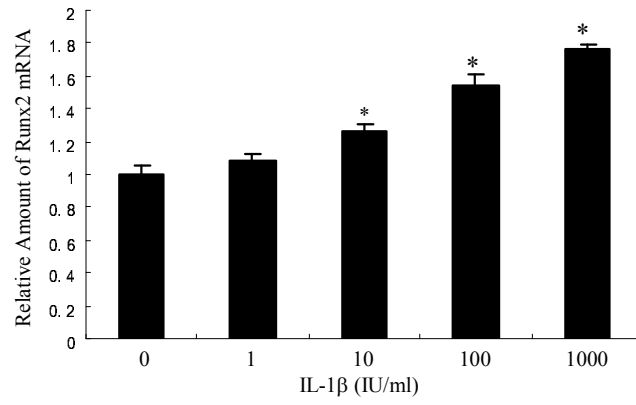
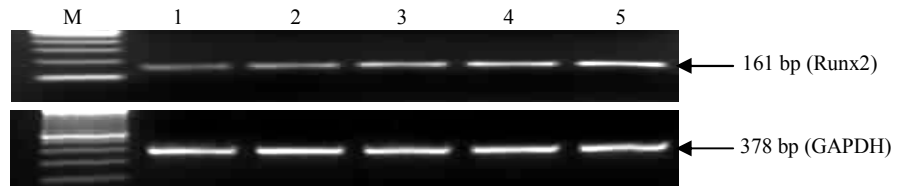


Figure 5.4. Concentration-dependent effects of IL-1 $\beta$  on Runx2 mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Runx2 mRNA in EVT<sub>s</sub> cultured in the presence of vehicle alone (lane 1) or increasing concentrations of IL-1 $\beta$  (1, 10, 100 or 1000 IU; lanes 2-5, respectively). A 100-bp ladder is shown in lane M with the size of the target cDNA indicated at the right. Representative photomicrographs of the resultant ethidium bromide-stained gels are presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 IU/ml control). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT cultures treated with vehicle alone (lane 1) or increasing concentrations of IL-1 $\beta$  (1, 10, 100 or 1000 IU; lanes 2-5, respectively) and probed with rabbit polyclonal antibodies against RUNX2 or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis as well as those from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 IU/ml control).

Figure 5.4

A



B.

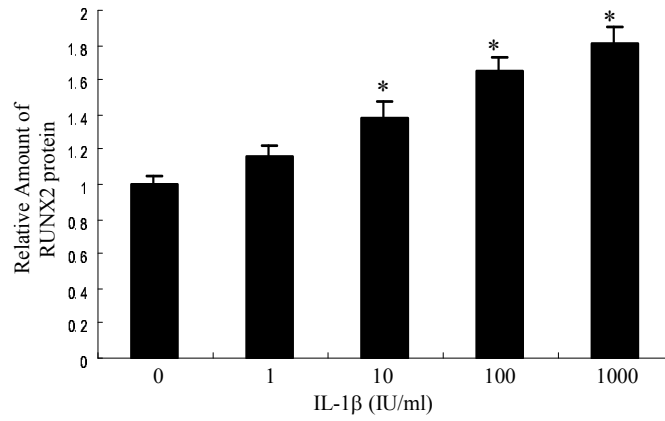
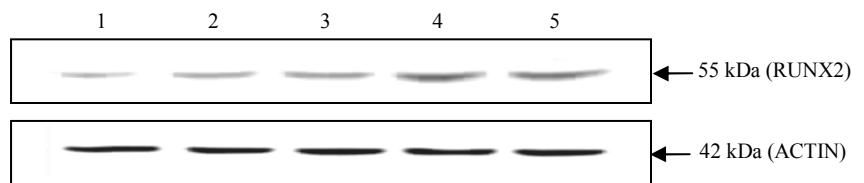


Figure. 5.5. Concentration-dependent effects of TGF- $\beta$ 1 on Runx2 mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Runx2 mRNA in EVT<sub>s</sub> cultured in the presence of vehicle alone (lane 1) or increasing concentrations of TGF- $\beta$ 1 (0.001, 0.01, 0.1, 1 or 10 ng/ml; lanes 2-6, respectively). A 100-bp ladder is shown in lane M with the size of the target cDNA indicated at the right. Representative photomicrographs of the resultant ethidium bromide-stained gels are presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 ng/ml control) in the graph. B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT<sub>s</sub> cultures treated with vehicle alone (lane 1) or increasing concentrations of TGF- $\beta$ 1 (0.001, 0.01, 0.1, 1 or 10 ng/ml; lanes 2-6, respectively) and probed with rabbit polyclonal antibodies against RUNX2 or human  $\beta$ -actin. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis as well as those from three other studies were standardized to the untreated control and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to 0 ng/ml control).

Figure 5.5

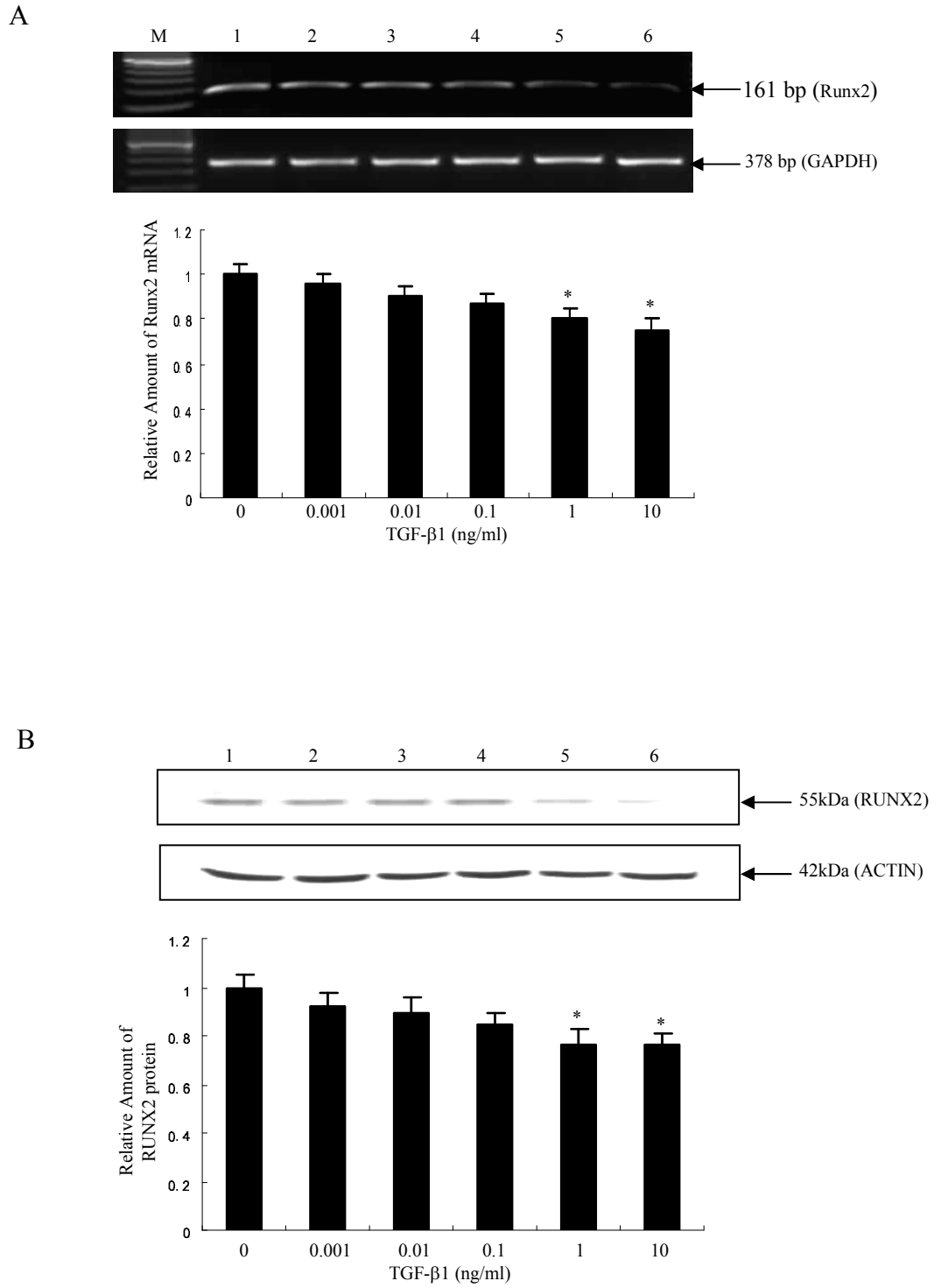


Figure 5.6. Attenuation of IL-1 $\beta$ -mediated increase in Runx2 mRNA and protein levels in EVT. A) Semi-quantitative RT-PCR analysis of Runx2 mRNA levels in EVT cultured with vehicle alone (lane 1), IL-1 $\beta$  alone (100 IU/ml; lane 2), IL-1 $\beta$  (100 IU/ml) plus 1 ug/ml of an anti-IL-1 $\beta$  antibody (lane 3) or IL-1 $\beta$  (100 IU/ml) plus 2 ug/ml of an anti-IL-1 $\beta$  antibody (lane 4) for 24 h. A 100 bp ladder is shown in lane M with the size of the target cDNA indicated at the right. A representative photomicrograph of the resultant ethidium bromide-stained gels is presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are presented as (mean  $\pm$  S.E.M., n = 4) in the bar graph (a,  $P < 0.05$  compared to untreated control; b,  $P < 0.05$  compared to cytokine alone). B) Representative fluorogram of a Western blot containing 30 ug of total protein extracted from corresponding EVT cultures treated with cultured with vehicle alone (lane 1), IL-1 $\beta$  alone (100 IU/ml; lane 2), IL-1 $\beta$  (100 IU/ml) plus 1 ug/ml of an anti-IL-1 $\beta$  antibody (lane 3) or IL-1 $\beta$  (100 IU/ml) plus 2 ug/ml of an anti-IL-1 $\beta$  antibody (lane 4). The blots were probed for RUNX2 and human  $\beta$ -actin (ACTIN). The data are presented as (mean  $\pm$  S.E.M., n = 4) in the bar graph (a,  $P < 0.05$  compared to untreated control; b,  $P < 0.05$  compared to cytokine alone).

Figure 5.6

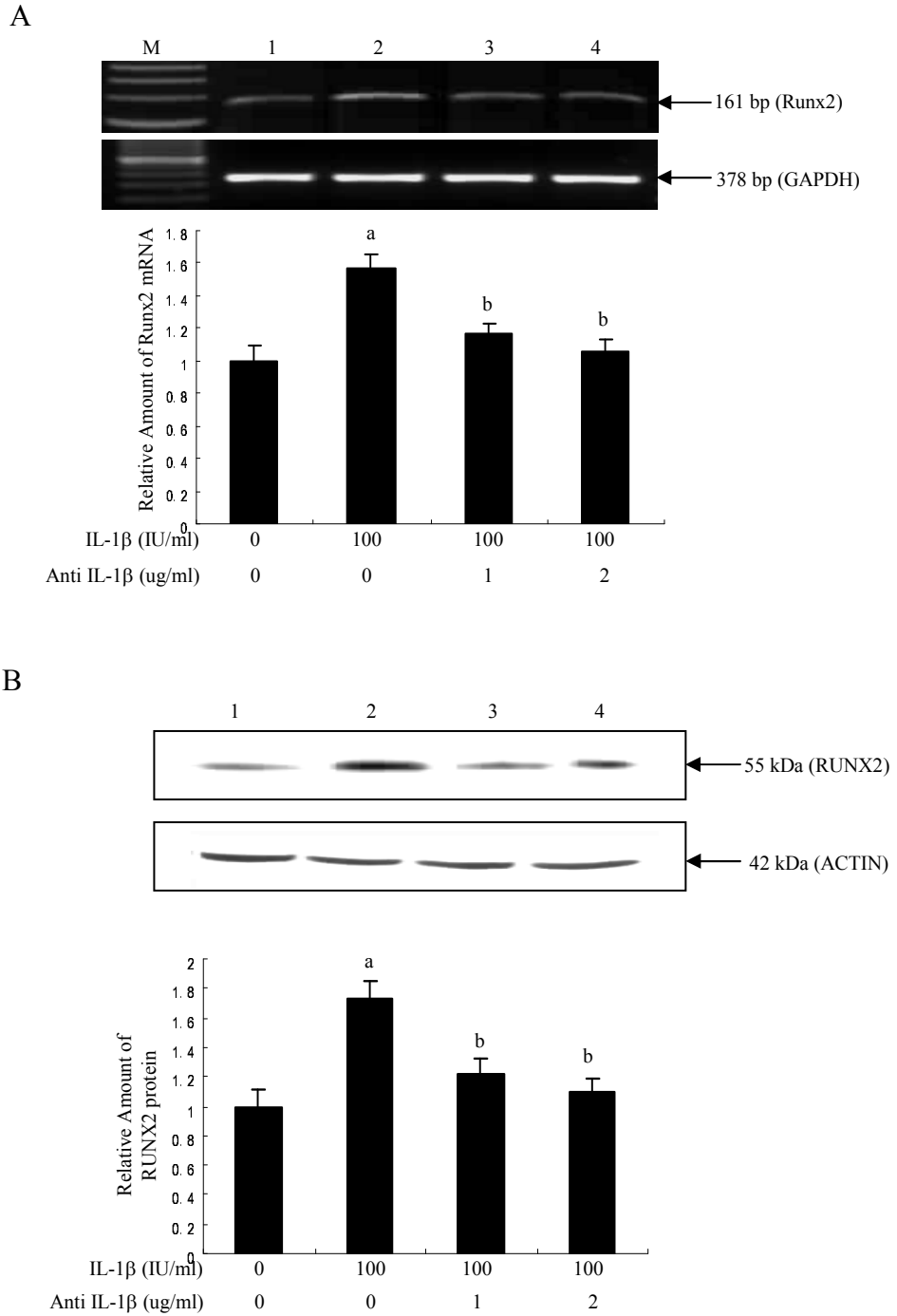




Figure 5.7. Attenuation of TGF- $\beta$ 1-mediated decreases in Runx2 mRNA and protein levels in EVT<sub>s</sub>. A) Semi-quantitative RT-PCR analysis of Runx2 mRNA levels in EVT<sub>s</sub> cultured with vehicle alone (lane 1), TGF- $\beta$ 1 alone (10 ng/ml; lane 2) or TGF- $\beta$ 1 (10 ng/ml) plus anti-TGF- $\beta$ 1 antibody (10  $\mu$ g/ml; lane 3). A 100 bp ladder is shown in lane M with the size of the target cDNA indicated at the right. A representative photomicrograph of the resultant ethidium bromide-stained gels is presented. Gels generated from this and three other independent experiments were analysed by densitometry and subjected to statistical analysis. The data are presented in the bar graph as (mean  $\pm$  S.E.M., n = 4; a,  $P < 0.05$  compared to untreated control; b,  $P < 0.05$  compared to cytokine alone). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from corresponding EVT cultures treated vehicle alone (lane 1), TGF- $\beta$ 1 alone (10 ng/ml; lane 2) or TGF- $\beta$ 1 (10 ng/ml) plus anti-TGF- $\beta$ 1 antibody (10  $\mu$ g/ml; lane 3). The blots were probed for RUNX2 and human  $\beta$ -actin (ACTIN). The data are presented in the bar graph as (mean  $\pm$  S.E.M., n = 4; a,  $P < 0.05$  compared to untreated control; b,  $P < 0.05$  compared to cytokine alone).

Figure 5.7

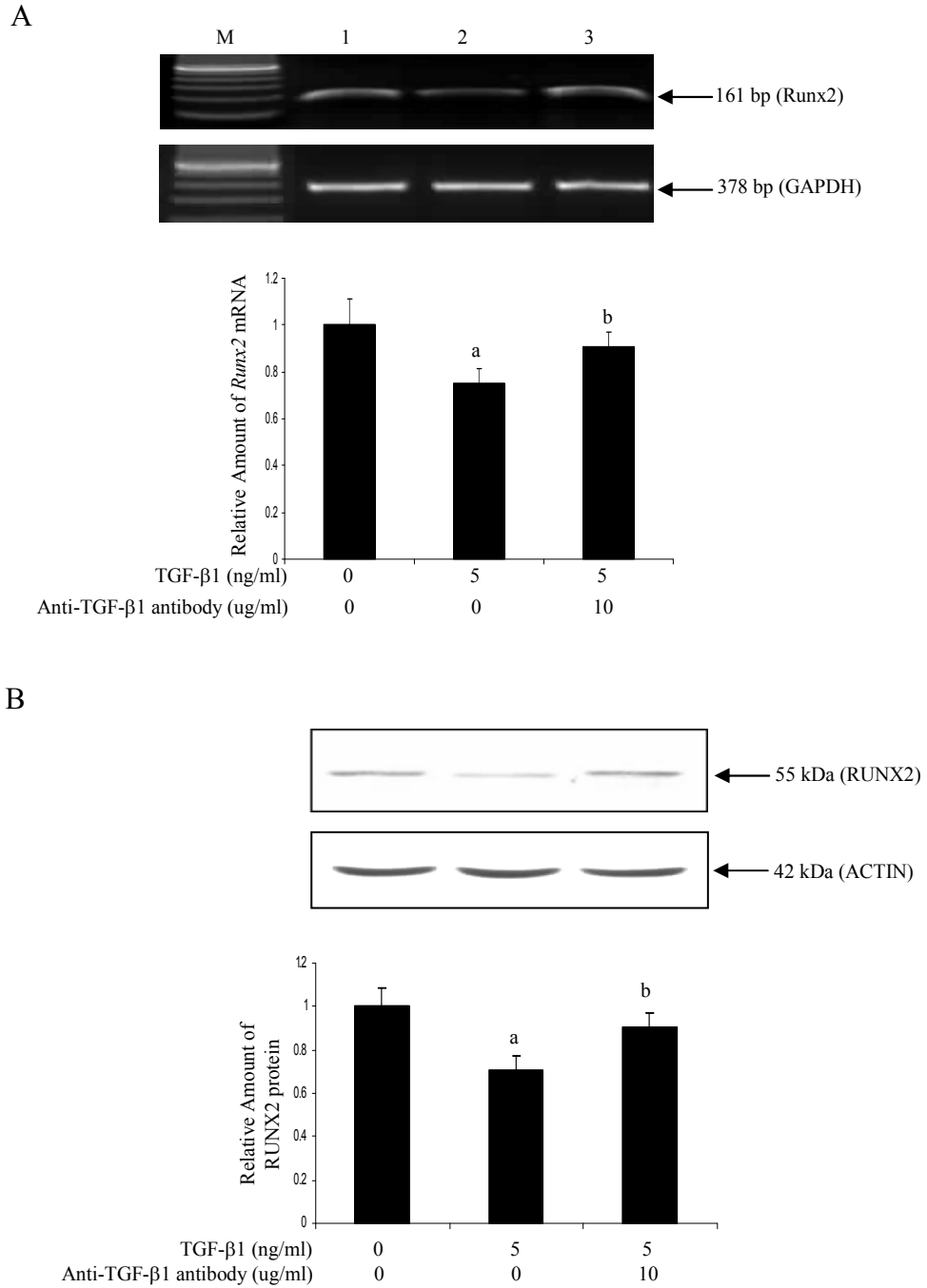
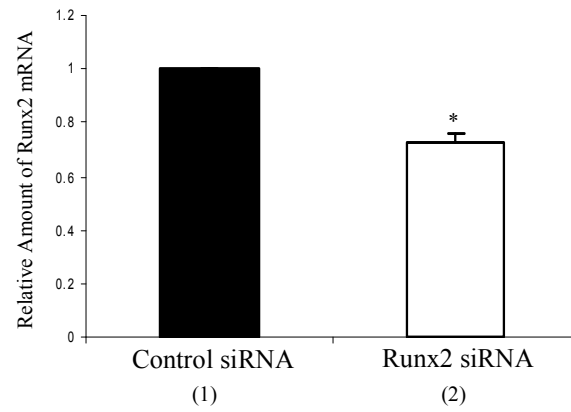


Figure 5.8. Effects of Runx2 siRNA on Runx2 mRNA and protein levels in HTR-8/SVneo cell cultures. A) Real-time RT-PCR analysis of Runx2 mRNA levels in cells transfected with a scrambled control siRNA (lane 1) or siRNA specific for Runx2 (lane 2) for 36 h. Values for Runx2 mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA). B) Representative fluorogram of a Western blot containing 30  $\mu$ g of total protein extracted from HTR-8/SVneo cells cultured with a scrambled control siRNA (lane 1) or siRNA specific for Runx2 (lane 2) for 36 h and probed with rabbit polyclonal antibodies against RUNX2 or human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for RUNX2 protein levels were normalized to the absorbance values obtained for  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis and from three other studies were standardized to the scrambled control siRNA and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA).

Figure 5.8

A



B

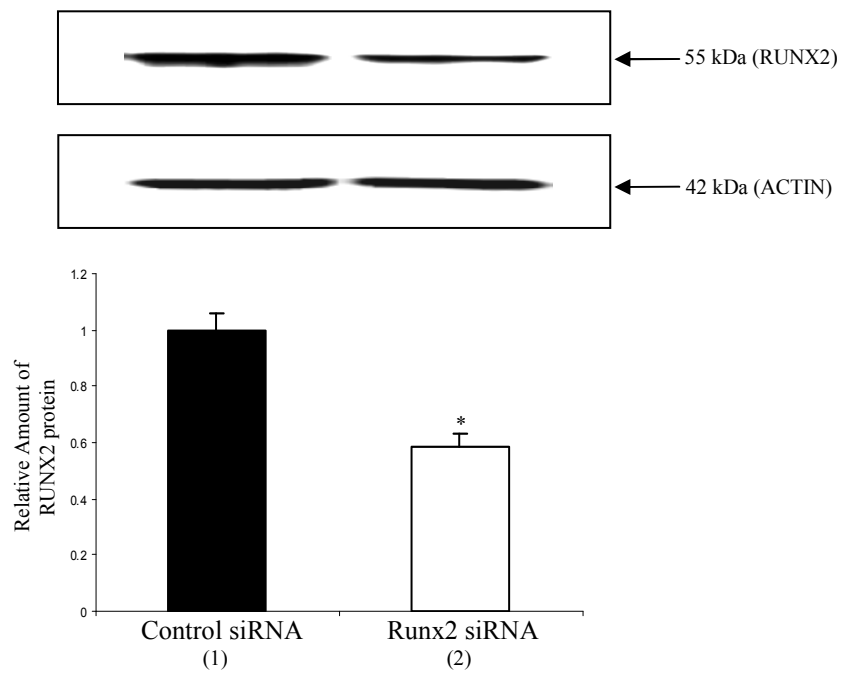
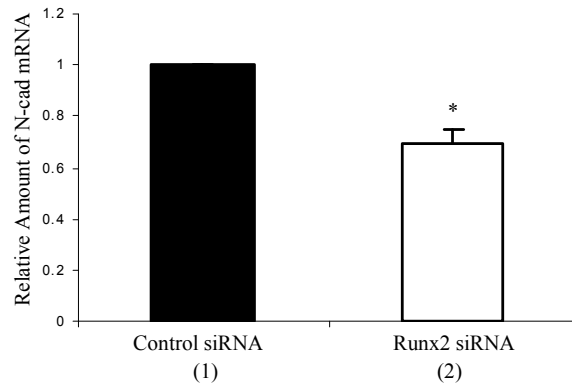


Figure 5.9. Effects of Runx2 siRNA on N-cad mRNA and protein levels in HTR-8/SVneo cell cultures. A) Real-time RT-PCR analysis of N-cad mRNA levels transfected with a scrambled control siRNA (lane 1) or siRNA specific for Runx2 (lane 2) for 36 h. Values for N-cad mRNA levels in each sample were normalized to the corresponding GAPDH mRNA levels. The data are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA). B) Representative fluorogram of Western blot containing 30  $\mu$ g of total protein extracted from HTR-8/SVneo cells cultured with a scrambled control siRNA (lane 1) or siRNA specific for Runx2 (lane 2) for 36 h and probed with mouse monoclonal antibody against N-CAD or rabbit polyclonal antibody against human  $\beta$ -actin. The Amersham ECL system was used to detect antibody bound to antigen. The resultant fluorograms were scanned and the absorbance values obtained for N-CAD protein levels were normalized to the absorbance values obtained for human  $\beta$ -actin (ACTIN) in the samples. The results derived from this analysis and from three other studies were standardized to the scrambled control siRNA and are represented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$  compared to scrambled control siRNA).

Figure 5.9

A



B

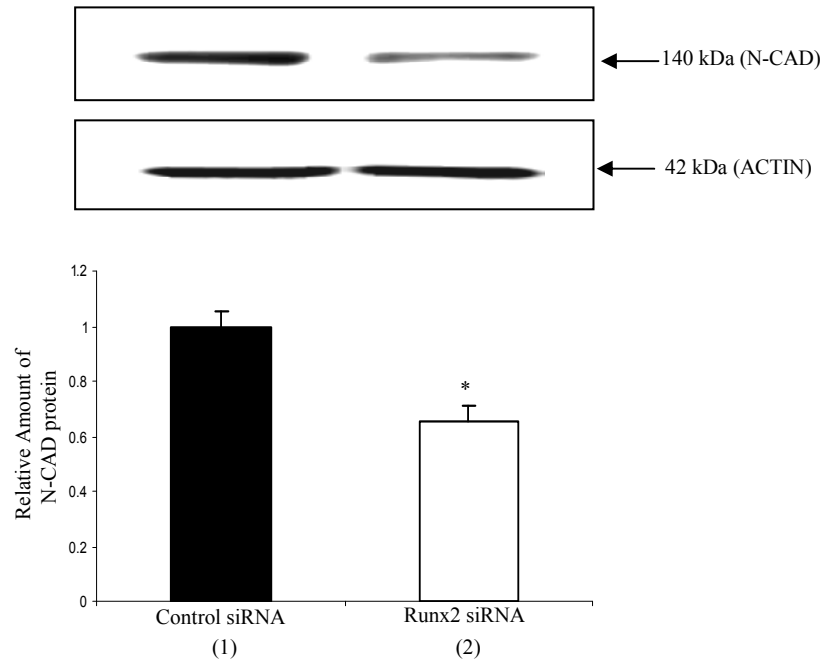
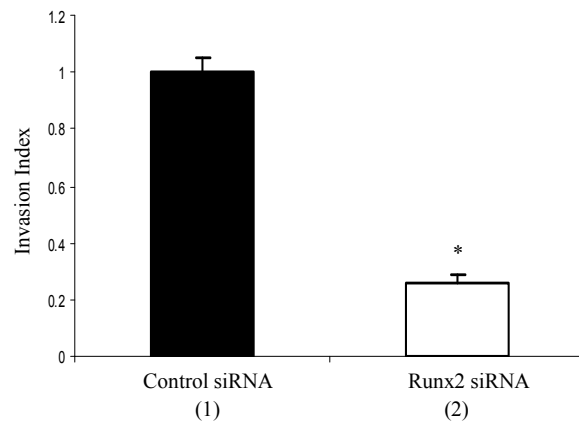


Figure 5.10. Reduced Runx2 levels decrease the invasive capacity of HTR-8/SVneo cells. HTR-8/SVneo cells were transfected with a scrambled control siRNA (lane 1) or siRNA specific for Runx2 (lane 2) for 24 h. The cells were then placed in the upper wells of Transwell invasion chambers. After a further 24 h of incubation, the porous membranes from the bottom of the Transwells were removed and fixed, stained and mounted upside-down on a glass microscope slide. Invasion was determined by counting the number of cells that invaded through the thin pre-coated layer of Matrigel on the top of the porous (8  $\mu$ m) membrane. Cells were visualized using a light microscope, and counted in three randomly selected fields of each membrane. Each cell line was plated in triplicate wells, and the experiment was repeated on three independent occasions. The results are presented (mean  $\pm$  S.E.M., n = 4) in the bar graph (\*,  $P < 0.05$ , compared to HTR-8/SVneo scrambled control siRNA).

Figure 5.10





### 5.3: Discussion and summary

Runx2 expression has been mostly characterized in bone tissue (Otto *et al.*, 1997), but it has also been found to be highly expressed in several other tissue types, including the testes (Ogawa *et al.*, 2000), mammary epithelium (Inman and Shore, 2003), endothelial cells (Sun *et al.*, 2001), and in prostate and breast tumours (Yeung *et al.*, 2002; Barnes *et al.*, 2003). In this study I have examined the expression of Runx2 in first-trimester human placenta tissue and in four human trophoblastic cell types: highly invasive primary EVT, the immortalized EVT cell line HTR-8 cells/SVneo, and the poorly invasive JEG-3 and BeWo choriocarcinoma cell lines. Runx2 was expressed in placental tissue, and the high levels of Runx2 expression in highly invasive EVT cells suggest that Runx2 plays a role in trophoblast invasion. Furthermore, the two choriocarcinoma cell lines, which are considered much less invasive, display significantly lower Runx2 expression.

It is known that IL-1 $\beta$  promotes human trophoblast invasion (Karmakar and Das, 2002) and in my studies it increased the expression of Runx2 in EVTs. It has also been shown that IL-1 $\beta$  increases the invasiveness of trophoblast cells, at least in part through the up-regulation of MMP-2 and MMP-9 (Karmakar and Das, 2002), which are key molecules in human trophoblast invasion (Staun-Ram *et al.*, 2004). While my results showed that IL-1 $\beta$  induces the expression of Runx2, I also demonstrated that TGF- $\beta$ 1 suppresses Runx2 expression in EVTs and this is important because TGF- $\beta$ 1 reduces the invasiveness of trophoblasts (Graham and Lala, 1991). TGF- $\beta$ 1 has been previously shown to decrease the functional activity of Runx2 by promoting an interaction between

SMAD3 and the Runx2 proteins in osteoblast cells (Alliston *et al.*, 2001). RUNX2 forms co-regulatory complexes with Smads and other co-activator and co-repressor proteins that are organized in subnuclear domains to regulate gene transcription. In addition, Runx2 can also mediate the responses of cells to hyperactive signalling pathways in tumours in response to TGF- $\beta$  and other growth factor signals. These observations strongly suggest that RUNX2 is a key molecule in regulating human trophoblastic cell invasion (Pratap *et al.*, 2006).

A siRNA knockdown of Runx2 in metastatic breast cancer cell lines reduced their invasive properties. In contrast, forced expression of Runx2 in non-metastatic breast cancer lines induced a three-fold increase in cellular invasion (Pratap *et al.*, 2005). In my loss-of-function study, I was able to demonstrate that a decrease in Runx2 expression is concomitant with a reduction in human trophoblast invasion.

Studies by Pratap *et al.* (2005) have provided experimental evidence that Runx2 can regulate the expression of MMP-9 and cellular invasion in breast cancer cell lines. They have demonstrated through chromatin immunoprecipitation that RUNX2 is recruited to the MMP-9 promoter. In other studies, Hazan *et al.* (2000) demonstrated that increased MMP-9 production in N-cad-expressing breast cancer cells occurred in response to fibroblast growth factor-2 and that this provided the cells with a greater ability to penetrate matrix protein barriers.

In my previous studies, I have demonstrated that N-CAD is a significant player in a molecular mechanism underlying the invasive capacity of EVT's *in vitro*. In addition, it has been previously reported that N-cad is a potential target of RUNX2 in embryonic bone tissue (Chua *et al.*, 2009). In this study, I have identified a role for Runx2 in

regulating N-cad in human trophoblastic cells. In support of this, when HTR-8/SVneo cells were transfected with siRNA against Runx2, N-cad expression was significantly reduced along with a reduction in the invasive ability of the cells.

In summary, my results provide evidence that RUNX2 is a key molecule in mediating human trophoblast invasion through regulating N-cad (Figure 5.11). However, further studies will be necessary to address the molecular interaction between RUNX2 and N-cad.

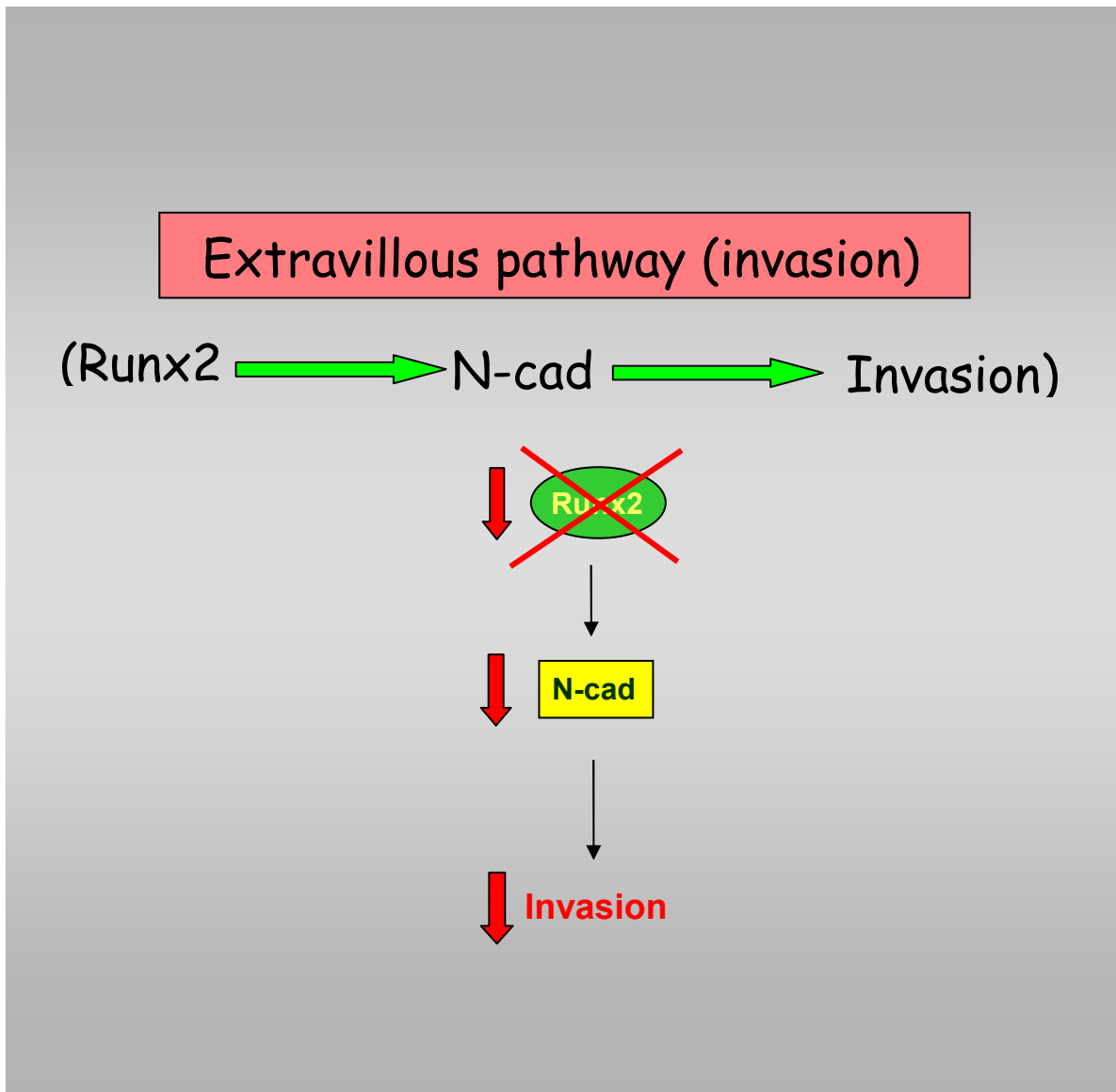


Figure 5.11. A schematic diagram of a proposed role of Runx2 in regulating N-cad-mediated differentiation of human trophoblastic cells. Silencing Runx2 expressing by siRNA strategy reduces N-cad expression level and reduces the invasion ability of human trophoblastic cells.

## CHAPTER 6: GENERAL DISCUSSION

### 6.1: Discussion

In my studies, I have investigated the roles of the transcription factors TWIST and RUNX2, and the cell adhesion molecules E-CAD and N-CAD, in the villous (non-invasive) and extravillous (invasive) pathways in human trophoblastic cells.

When studying the terminal differentiation of human cytotrophoblasts *in vitro*, I used both primary cultures of human trophoblasts and human trophoblastic cell lines. There are both advantages and disadvantages when using these cell cultures. The advantage of using primary cell cultures is their close biological and morphological proximity to the human placenta. However, there is a number of limitations to these primary cell cultures. Due to limited access to term placental tissues, I switched to using the BeWo trophoblastic cell line in order to study the cellular and molecular mechanisms in the villous pathway. As previously mentioned, BeWo cells have been widely used to study syncytialization in human trophoblasts in the presence of 8-bromo cAMP (Seamon *et al.*, 1981; Wice *et al.*, 1990). Furthermore, the advantages of using a cell line include their immortal and consistent characteristics, cell purity, and commercial availability. To investigate the extravillous pathway, I used primary EVT<sub>s</sub> for regulatory studies with cytokines such as TGF- $\beta$ 1 and IL-1 $\beta$ , because HTR-8/SVneo cells do not respond to TGF- $\beta$ 1 treatment in my experiments and as reported by another group (Graham *et al.*, 1993). As for the functional studies, I chose to use HTR-8/SVneo cells because these cells are easier to transfect and their characteristics are more uniform, as mentioned earlier.

My studies have provided a greater understanding of the cellular and molecular mechanisms that mediate the formation and organization of the human placenta, and demonstrate that Twist can regulate and promote cellular fusion (Figure 6.1). In addition, my studies have suggested a role for Twist, Runx2 and N-cad in regulating EVT invasion (Figure 6.2).

In the first part of my studies, I examined the expression of Twist and E-cad during the terminal differentiation and fusion of BeWo cells, a human trophoblastic cell line. I observed Twist was up-regulated and E-cad was down-regulated during the differentiation and fusion processes in human trophoblastic cells. In gain- or loss-of-function studies, I have shown that increasing Twist expression promotes terminal differentiation and fusion of human trophoblastic cells through down-regulation of E-cad expression.

In the second part of my studies, I investigated whether Twist and N-cad may play important roles in human trophoblast cell invasion. I first examined the expression of Twist and N-cad in highly invasive EVTs and the poorly invasive JEG-3 and BeWo human trophoblastic cell lines. My results have revealed that Twist and N-cad are highly expressed in highly invasive EVTs but the expression of both is significantly lower in the poorly invasive human trophoblastic cell lines.

Next, I determined the regulatory effects of IL-1 $\beta$  and TGF- $\beta$ 1, two cytokines known to control trophoblast invasion (Graham and Lala, 1991; Karmakar and Das, 2002), on primary cultures of EVTs. I found a differential regulation of Twist expression. Twist expression increases after IL-1 $\beta$  treatment in a time- and concentration-dependent manner.

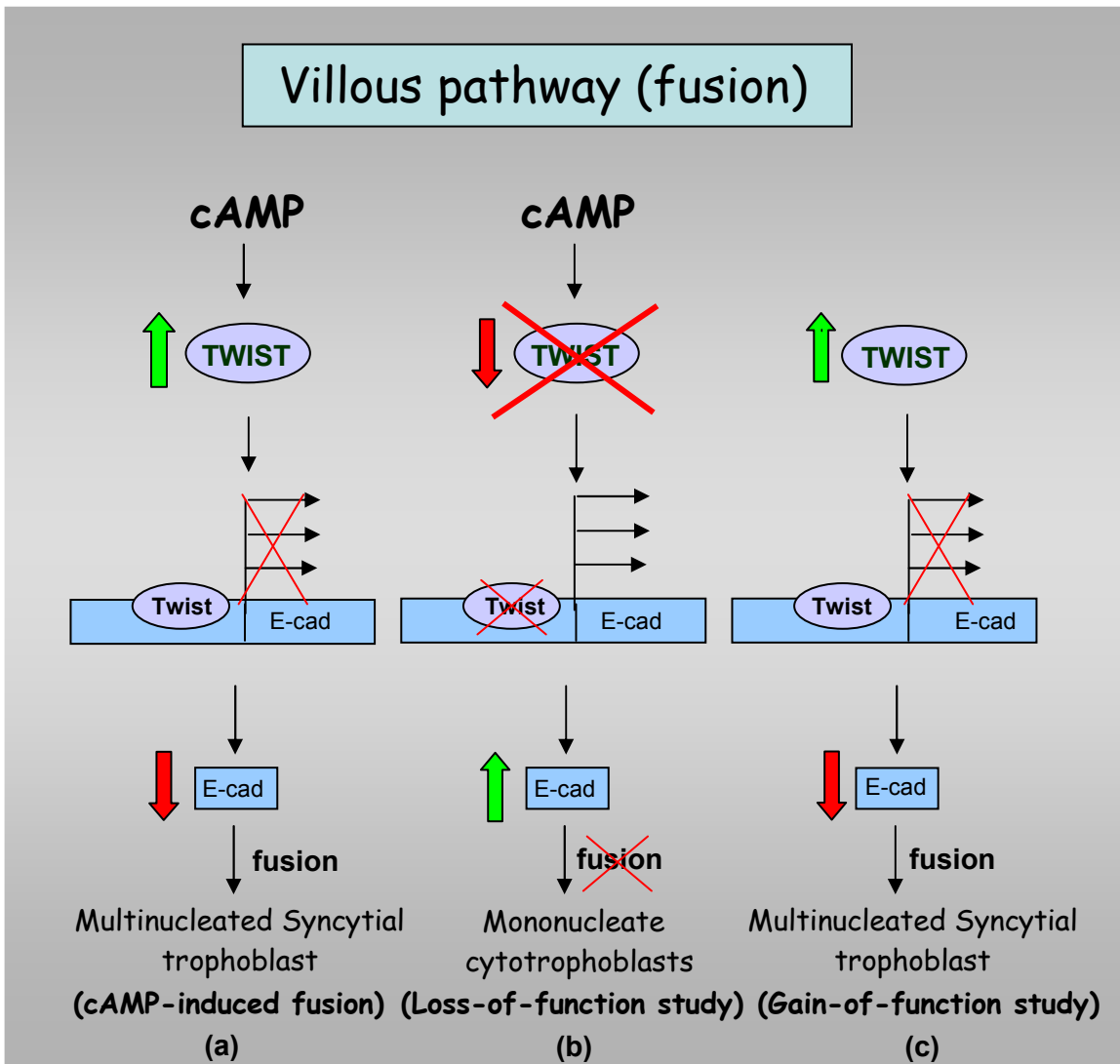


Figure 6.1. A schematic diagram of a proposed role of Twist in regulating terminal differentiation and fusion of human trophoblastic cells. (a) cAMP up-regulates TWIST levels and down-regulates E-cad expression to promote the formation of syncytial trophoblast. (b) Silencing Twist expression inhibited TWIST levels from up-regulated and E-cad expression from down-regulated in cAMP-induced BeWo cells. These cells remain as mononucleate cytotrophoblasts. (c) Stable transfection with Twist expression vectors in BeWo cells reduces E-cad expression and promotes the formation of syncytial trophoblast.

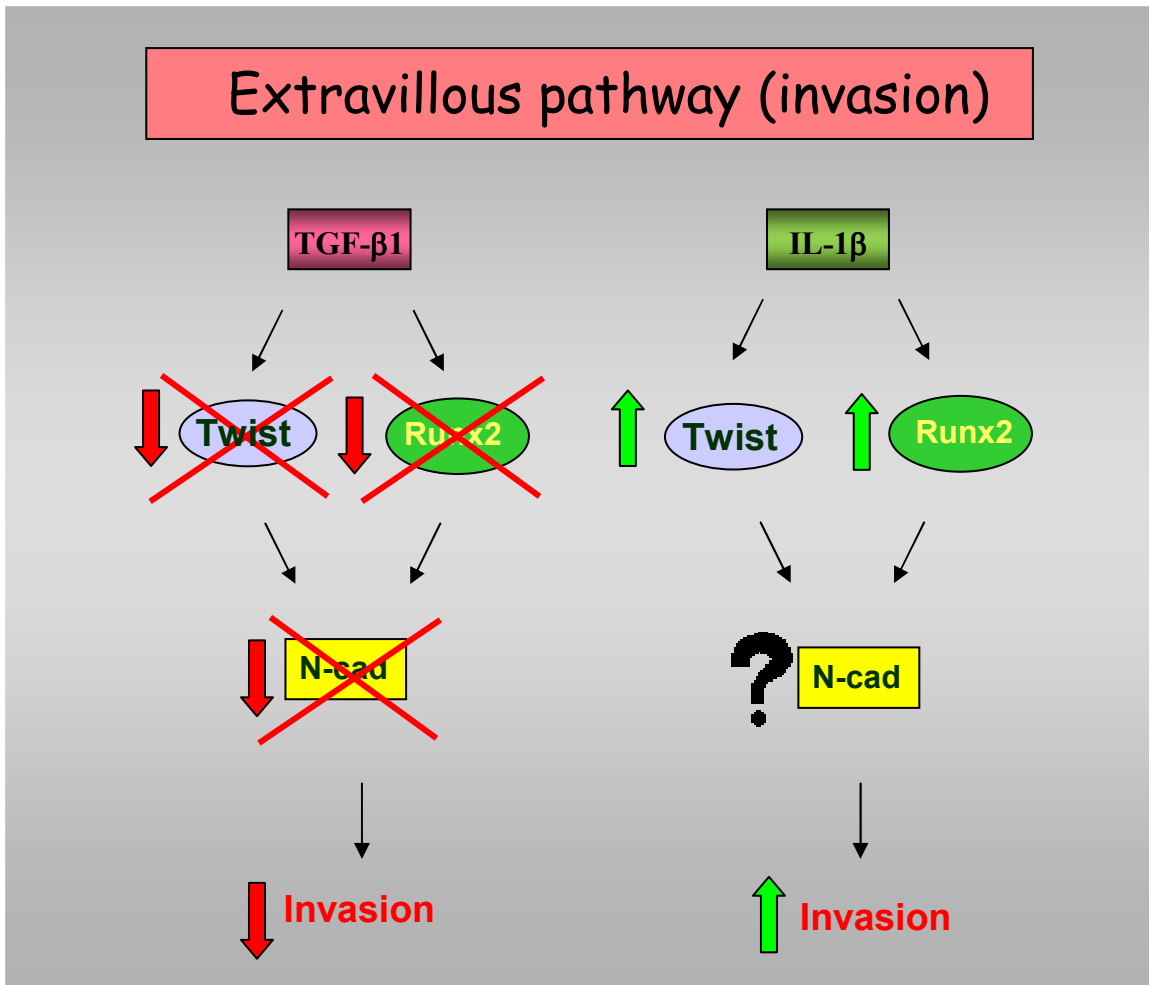


Figure 6.2. A schematic diagram of the proposed roles of Twist, Runx2 and N-cad in regulating the invasive ability of human trophoblastic cells. TGF-β1 down-regulates Twist and Runx2 expression and reduces the invasion ability of EVT. IL-1β up-regulates Twist and Runx2 expression and increases the invasion ability of EVTs. Silencing Twist or Runx2 expression reduces N-cad expression and reduces the invasion ability of HTR-8/SVneo cells. Silencing N-cad expression reduces the invasion ability of HTR-8/SVneo cells.



In contrast, Twist expression with TGF- $\beta$ 1 treatment decreases in a time and concentration-dependent manner. However, IL-1 $\beta$  and TGF- $\beta$ 1 have no effects upon N-cad expression, suggesting that other growth factors may be involved in regulating N-cad expression in human EVTs.

I continued to determine a role for Twist in human trophoblast invasion by performing a gain-of-function study using JEG-3 cell line. Although Twist mRNA level increased, the protein level remains unchanged. Therefore, I could not determine a role for Twist in trophoblast invasion in these cell models.

I then took an opposite approach by using a loss-of-function study to silence Twist expression. This demonstrated that down-regulation of Twist reduced the invasive capacity of the EVT cell line and the expression of N-cad, suggesting that Twist promotes trophoblast invasion *via* N-cad.

Next, I performed a loss-of-function study by utilizing a siRNA strategy to silence N-cad expression to investigate a role for this CAM in human trophoblastic cell invasion. My results show that by silencing N-cad expression the trophoblastic cells became significantly less invasive. I continued to determine a functional role for N-CAD in these trophoblastic cells by using a function-perturbing antibody against the extracellular binding domain of N-CAD. This antibody effected a significant reduction in trophoblastic cell invasion, thus confirming a functional role of N-CAD in human trophoblastic cells *in vitro*.

I continued to investigate whether Runx2 plays important roles in human trophoblast cell invasion. First, I demonstrated that Runx2 is highly expressed in highly invasive EVTs, but it is present at significantly lower levels in poorly invasive JEG-3 and BeWo

trophoblastic cell lines. Next, I investigated whether IL-1 $\beta$  and TGF- $\beta$ 1 were able to respectively promote and repress Runx2 mRNA and protein levels in EVT<sub>s</sub>. I found that IL-1 $\beta$  significantly increases Runx2 expression, while TGF- $\beta$ 1 significantly reduces Runx2 expression. Taken together, these observations support my hypothesis that Runx2 plays an important role in human trophoblast invasion. In my loss-of-function study, silencing Runx2 expression results in significant reduction on N-cad expression and reduces the invasive ability of HTR-8/SVneo cell.

Membrane fusion plays a critical role in development. For example, it mediates the fertilization of the egg and sperm (Wilson and Snell, 1998), and the formation of myotubes from the mononucleate myoblasts in the embryo (Nadal-Ginard, 1987). In addition, during human placentation, it mediates the formation of the syncytial trophoblast from the mononucleate cytotrophoblasts (Kliman *et al.*, 1986). Cell fusion not only brings about morphological changes in these trophoblastic cells, but also influences the physiology of the cells. For instance, the formation of the syncytial trophoblast plays a critical role in the onset of placental hormone production (Hoshina *et al.*, 1982; Cronier *et al.*, 1994).

Fox (1964) demonstrated a decrease in the thickness of the syncytial trophoblast layer and an increase in the number of villous cytotrophoblasts in human placental specimens from pregnancies complicated by intrauterine hypoxia. A reduction in the formation of syncytial trophoblast structures in cultures of villous cytotrophoblasts isolated from the term placenta was observed in patients suffering from preeclampsia, a condition in pregnancy characterized by a sharp rise in blood pressure together with large amounts of protein in the urine (Pijnenborg *et al.*, 1996).

It has been well documented that cell differentiation and morphogenesis depend partly on the regulated expression of cell adhesion molecules. The commonly known cell adhesion molecules that play a role in terminal differentiation and fusion of human cytotrophoblasts include CAD-11 (MacCalman *et al.*, 1997) and E-CAD (Coutifaris *et al.*, 1991). High levels of E-CAD were immunolocalized to the surface of BeWo cells before they undergo terminal differentiation and fusion, and E-CAD is needed for the cells to undergo cell aggregation prior to cell fusion (Coutifaris *et al.*, 1991). Down-regulation of E-cad expression has been shown to be necessary to promote the formation of syncytial trophoblast (Coutifaris *et al.*, 1991; Getsios *et al.*, 2003). In a similar manner, E-cad expression was also down-regulated during the formation of multinucleated osteoclasts from bone marrow mononucleate cells in mice (Suda *et al.*, 1992).

Several studies have identified the ability of growth factors to regulate the terminal differentiation and fusion of villous cytotrophoblasts. One example would be epidermal growth factor (EGF) that is produced by both decidual cells and trophoblastic cells at the maternal-fetal interface (Hofmann *et al.*, 1992; Morrish *et al.*, 1998; Leach *et al.*, 1999). Blocking EGF receptor function using an anti-EGF-receptor monoclonal antibody increases E-cad expression in primary cultures of human trophoblastic cells (Rebut-Bonneton *et al.*, 1993; Al Moustafa *et al.*, 1999). This suggests that EGF can down-regulate E-cad expression in human trophoblastic cells. EGF treatment of A431 cells, an epidermoid cancer cell line, has been shown to increase cAMP accumulation induced by forskolin (Ball *et al.*, 1990). In addition, cAMP analog has shown to increase MAPK-1/3

and cAMP response element-binding protein (CREB) phosphorylation in BeWo cells (Maymo *et al.*, 2010).

TWIST, a helix-loop-helix transcription factor that has long been implicated in embryonic morphogenesis, also promotes epithelial-mesenchymal transition (EMT) through the down-regulation of E-cad in a wide range of cancer cells (Yang *et al.*, 2004, Kwok *et al.*, 2005, Yuen *et al.*, 2007). Yang *et al.* (2004) demonstrated that Twist transcriptionally represses E-cad in human breast cancer cells through direct interaction with an E-Box present in the promoter region of E-cad. As mentioned before, I did not determine the actual mechanism by which up-regulated Twist expression effects down-regulated E-cad expression in these trophoblastic cells. However, it is possible that TWIST represses E-cad through direct contact with the E-box present in the promoter region of E-cad during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro*. In order to examine whether the repression of E-cad by TWIST in my studies is determined by the E-box located within the promoter region, luciferase reporter gene assays will have to be carried out to assess the transcription efficiency of the E-cad promoter with an E-box mutation. My studies are the first to investigate a role for the Twist gene in terminal differentiation of human cytotrophoblasts *in vitro*. Collectively, the observations from my studies suggest that Twist and E-cad, which mediate EMT processes in cancer cells, can also influence the terminal differentiation and fusion of human trophoblasts. Additional studies will be needed to identify the underlying mechanisms that increase Twist expression in the human trophoblast differentiation.

Increased expression of N-cad promotes the motility and invasion of carcinoma cells (Nienam *et al.*, 1999; Cavallaro *et al.*, 2002; Hazan *et al.*, 2000). Studies have suggested that N-cad-expression in cancer cells may promote homophilic interactions with N-cad expressing tissues such as endothelia and stroma (Hazan *et al.*, 2000). For example, N-CAD mediates the transmigration of melanomas through the vascular endothelium (Sandig *et al.*, 1997). Although it is tempting to speculate that N-cad expression in EVT<sub>s</sub> may facilitate adhesion with the endometrial stroma or the maternal endovascular cells to facilitate the remodelling of the endothelial lining, my single cell model could not confirm this.

Besides facilitating homophilic cell adhesion between cancer cells and normal cells, N-cad is also believed to promote cell invasion through cell signalling events (Hazan *et al.*, 2004). It has been reported that in breast cancer cell invasion, there is an interaction between N-CAD and fibroblast growth factor receptor 1 (FGFR1) at the cell surfaces to form an extracellular complex. This complex is formed by the interaction between the extracellular domain of N-CAD and immunoglobulin domains 1 and 2 of FGFR-1, which eventually stabilizes FGFR-1 and leads to prolonged mitogen-activated protein kinase (MAPK) activation (Kim *et al.*, 2000; Suyama *et al.*, 2002).

Hazan *et al.* (2004) proposed that while N-cad promotes homophilic cell-adhesive mechanisms, E-cad controls tumour progression. Previous studies have reported that down-regulation of E-cad is necessary for cancer cells to become invasive (Onder *et al.*, 2008). However, preliminary data from our laboratory (Alex Beristain, unpublished data) show that the exogenous expression of N-cad in JEG-3 cells leads to increased invasion without altering the expression of E-cad. Similarly, Nieman *et al.* (1999) have reported

that N-cad plays a dominant role over the high expression of E-cad in promoting breast cancer cell invasion. In contrast, exogenous expression of E-cad resulted in decreased invasiveness and reduction of N-cad expression in tumour cells expressing endogenous N-cad (Yanagisawa and Anastasiadis, 2006). Indeed, the molecular mechanisms in which N-cad and E-cad regulate invasion remain unclear, probably due to the differences in cell types.

TWIST is able to increase N-cad expression in a variety of cancer cells (Alexander *et al.*, 2002; Rosivatz *et al.*, 2002). Although the precise mechanism by which Twist regulates N-cad in human trophoblastic cells is unknown, others have found that the effect of TWIST on the induction of N-cad mRNA requires an E-box located within the first intron of the N-cad gene (Alexandra *et al.*, 2006).

RUNX2 is a scaffolding transcription factor, which has been well documented to play a role in osteoblast differentiation (Otto *et al.*, 1997). Runx2-null mutations in mice result in severe bone deficiency as well as hypothyroidism, a disease caused by insufficient production of thyroid hormones (Harada and Roden, 2003; Endo and Kobayashi, 2010). Runx2 is phosphorylated and can be activated by the MAPK pathway (Xiao *et al.*, 2000). Runx2 is known to play key roles in angiogenesis (Zelzer *et al.*, 2001), and vascular invasion of bone and is highly expressed in endothelial cells (Mundlos, 1999; Enomoto *et al.*, 2000). Runx2 has been termed an oncogene and it is highly expressed in different types of cancer cells, including breast and prostate cancer cells (Barnes *et al.*, 2004; Javed *et al.*, 2005, Pratap *et al.*, 2006b). These studies have provided additional support that Runx2 play an important role in regulating human trophoblast invasion. My results are also the first to show a regulatory linkage between

Runx2 and N-cad. However, further studies will be necessary to identify the molecular mechanisms on how Runx2 regulates N-cad expression.

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is a secreted protein that controls cell growth, cell proliferation, cell differentiation and apoptosis (Ghadami *et al.*, 2000; Vaughn *et al.*, 2000). TGF- $\beta$ 1 has been assigned major regulatory roles in placental development and in decidualization of the endometrial stroma (Godkin and Dore, 1998; Karmakar and Das, 2002). This growth factor binds to a type II receptor dimer, which recruits and phosphorylates a type I receptor dimer to form a hetero-tetrameric complex (Wrana *et al.*, 1992). The type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs) to form R-SMAD/coSMAD (e.g. SMAD4) complexes in the nucleus to regulate targeted gene expression (Souchelnytskyi *et al.*, 2001; Feng and Derynck, 2005). TGF- $\beta$  is able to differentially regulate Twist expression in a variety of cell types (Leu *et al.*, 2008; Mori *et al.*, 2009; Murakami *et al.*, 2010) and to decrease RUNX2 binding to DNA (Komori, 2006).

Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a pro-inflammatory cytokine that plays a key regulatory role in the establishment of pregnancy (Salamonsen *et al.*, 2000, 2003; Fazleabas *et al.*, 2004). IL-1 $\beta$  can bind to type 1 IL-1 receptor protein, IL-1 binding activates IL-1 receptor associated kinase-1 and -2 (IRAK-1 and -2), IRAK-1 then recruits TNF receptor associated factor 6 (TRAF6) to the IL-1 receptor complex and activates two pathways, one leading to the map-erk kinase (MEK)/ c-Jun N-terminal kinases (JNK) signalling system and the other leading to nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation (Muzio *et al.*, 1997; Allan and Rothwell, 2001; Wang *et al.*, 2001). Several NF- $\kappa$ B targets include Twist, snail1, zeb1 (Min *et al.*, 2008).

## **6.2: Summary and Conclusions**

My studies are the first to find that Twist is up-regulated and E-cad is down-regulated during the terminal differentiation and fusion of human trophoblastic cells. By using gain- and loss-of-function studies, I was able to demonstrate that Twist is a key regulator of E-cad in these trophoblastic cells. My results also show that Twist regulates cadherin-mediated morphological and functional differentiation of human trophoblastic cells. The second part of my thesis describes studies that identify a role for Twist in extravillous cytotrophoblasts. These demonstrated that Twist promotes human trophoblastic cell invasion through down-regulating N-cad expression. In a loss-of-function study in which N-cad expression was silenced, there was a significant reduction of trophoblastic cell invasion. Finally, I was able to demonstrate that Runx2, which is known to regulate cancer invasion, also regulates human trophoblastic cell invasion. In addition, silencing Runx2 expression caused a significant down-regulation of N-cad expression.

Although the molecular mechanisms involved in the terminal differentiation of human trophoblast are rather complex, my findings help in furthering the understanding of many diseases that are linked to human placentation.



### **6.3: Future directions**

A successful human pregnancy depends upon mononucleate cytotrophoblasts entering one of two distinct and mutually exclusive pathways. Villous cytotrophoblastic cells proliferate and differentiate by fusion to form the outer syncytial trophoblast, or enter the extravillous pathway to form highly invasive extravillous cytotrophoblasts. However, the molecular mechanisms involved in determining which pathway the mononucleate cytotrophoblasts will enter remain to be elucidated. Studies have shown that the interaction between TWIST and RUNX2 involves unique domains in these proteins, the twist box and the RUNX2 DNA binding domain (Lian *et al.*, 2004), and it will be necessary to determine if the fate of mononucleate cytotrophoblast is differentially or co-ordinately regulated by Twist and Runx2 expression.

#### **6.3.1: Investigate the regulation and function of Runx2 in human trophoblast cell fusion**

First, it will be necessary to examine Runx2 mRNA and protein expression levels in BeWo choriocarcinoma cells undergoing terminal differentiation and fusion in response to the secondary intracellular signalling molecule 8-bromo-cAMP, by using semi-quantitative RT-PCR and Western blotting, respectively. Subsequently, gain- or loss-of-function studies could be carried out using these cells and either a mammalian expression vector containing a cDNA encoding Runx2 or siRNA specific for this transcription factor, respectively. In addition to examining subsequent Runx2 and E-cad mRNA and protein expression levels in these cell cultures, the presence or absence of multinucleated syncytia

will be confirmed by indirect immunofluorescence using antibodies directed against RUNX2, E-CAD or DESMOPLAKIN, a cellular marker of mononucleate cytotrophoblasts.

### **6.3.2: Investigate whether the inter-related expression of Runx2 and Twist determines the differentiation pathway of mononucleate cytotrophoblasts**

My preliminary findings demonstrate that Twist expression levels correlate with Runx2 expression levels in human trophoblastic cell invasion. Further investigation is needed into the regulatory effects between Twist and Runx2 in the formation of the multinucleated syncytial trophoblast of the human placenta. The co-coordinated or differentially regulated expression of Twist and Runx2 in human trophoblastic cells could be the determining factor for whether the mononucleate cytotrophoblasts enter either the villous or invasive pathway.

In future studies, I would investigate the relationship(s) between Runx2 and Twist in terminal differentiation of human trophoblastic cells, either by transfecting a siRNA for Runx2 or Twist into BeWo cells that can undergo terminal differentiation and fusion in response to the secondary intracellular signalling molecule 8-bromo-cAMP. Semi-quantitative RT-PCR and Western blotting can be used to examine Runx2 and Twist mRNA and protein levels in these trophoblastic cells.

### **6.3.3: Immunolocalization of TWIST and RUNX2 at the human maternal-fetal interface during pregnancy**

If TWIST and RUNX2 in fact play critical role in formation, maintenance, and/or function of the maternal-fetal interfacen then it would be important to be confirm that these proteins are indeed localized here during one or more stages of pregnancy. Tissue sections will be prepared from archived permanent paraffin blocks containing maternal-fetal tissues (n=3) obtained during the first trimester of pregnancy. These tissue sections will be immunostained using a commercially available polyclonal antibody directed against human TWIST or RUNX2. A nonspecific isotype-matched antibody would served as an appropriate negative control for these experiments.

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APPENDIX



The University of British Columbia  
Office of Research Services  
Clinical Research Ethics Board –  
Room 210, 828 West 10th Avenue,  
Vancouver, BC V5Z 1L8

## ETHICS CERTIFICATE OF FULL BOARD APPROVAL: RENEWAL

<b>PRINCIPAL INVESTIGATOR:</b> Colin D. MacCalman	<b>DEPARTMENT:</b> UBC/Medicine, Faculty of/Obstetrics & Gynaecology	<b>UBC CREB NUMBER:</b> H06-70260
<b>INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:</b>		
Institution	Site	
N/A Other locations where the research will be conducted: N/A		
<b>CO-INVESTIGATOR(S):</b> Hua Zhu York HUNT Ng Fatemeh K. Khatibi		
<b>SPONSORING AGENCIES:</b> - Canadian Institutes of Health Research (CIHR) - "Structure, Function and Regulation of the Cadherin-11 Complex in the Human Placenta" - Canadian Institutes of Health Research (CIHR) - "Structure, function and regulation of the cadherin-11 complex in the human placenta"		
<b>PROJECT TITLE:</b> Structure, Function and Regulation of the Cadherin-11 Complex in the Human Placenta		

**THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: January 27, 2010**

The UBC Clinical Research Ethics Board Chair or Associate Chair, has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.	
<b>DOCUMENTS INCLUDED IN THIS APPROVAL:</b> N/A	<b>APPROVAL DATE:</b> February 17, 2009
<b>CERTIFICATION:</b> <b>In respect of clinical trials:</b> 1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations. 2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices. 3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed	

*consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.*

The UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

*Approval of the Clinical Research Ethics Board by :*

**Dr. Gail  
Bellward,  
Chair**