CHARACTERIZATION OF THE MOLECULAR MECHANISMS UNDERLYING THE CADHERIN-MEDIATED DIFFERENTIATION OF HUMAN TROPHOBLASTIC CELLS

by

YIN WAN KWOK

B.Sc. The University of Hong Kong, 2002

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

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

DECEMBER 2004

© Yin Wan Kwok, 2004
The majority of the biological functions of the human placenta are performed by the syncytial trophoblast layer, a multinucleated cell that is formed by the terminal differentiation and fusion of the underlying layer of mitotically active, mononucleate cytotrophoblasts. This key step in the formation and organization of the placenta is mediated by a decrease in E-cadherin (E-cad) and a concomitant increase in the levels of Cadherin-11 (cad-11) expressed on the surface of differentiating trophoblastic cells. Similarly, aberrant expression of these two cadherin subtypes has also been associated with the development of persistent trophoblastic disease in vivo. To identify the mechanisms underlying the switch in cadherin expression in human trophoblasts, we examined the mRNA levels and the protein levels of Snail and Slug, 2 zinc finger transcription repressors that regulate E-cad expression, and Twist, a transcription factor associated with tumor metastasis, in BeWo choriocarcinoma cells undergoing differentiation and fusion in response to the intracellular secondary messenger, cAMP. Quantitative Competitive Polymerase Chain Reaction (QC-PCR) analysis revealed significant declines in E-cadherin mRNA levels in BeWo choriocarcinoma cells after 12h of culture in the presence of cAMP. The levels of this mRNA transcript continued to decline until the termination of these studies at 48h. Western blot analysis and immunostaining also demonstrated a significant decrease in the protein levels of E-cadherin in response to cAMP. A significant and continuous increase in Snail mRNA levels and protein levels were detected in these trophoblastic cells over time in culture with cAMP using QC-PCR and Western Blot. Intense immunostaining for Snail expression was also detected in the nuclei of cultured BeWo cells. However, the levels of
the mRNA transcript encoding Slug remained relatively low and constant, at least at the time points examined in these studies. There was also a significant increase in Twist mRNA levels and protein levels as the BeWo cells underwent terminal differentiation and fusion. Collectively, these observations suggest that Snail and Twist regulate the switch in cadherin subtypes expressed on the surface of human trophoblastic cells, a cellular event underlying the formation of multinucleated syncytium in vivo and in vitro. Future studies will examine the effects of loss-, or gain-of-function of Snail and Twist on the terminal differentiation and fusion of human trophoblastic cells in vitro.
TABLE OF CONTENTS

ABSTRACT.................................................................................................................. ii
TABLE OF CONTENTS..................................................................................................... iv
LIST OF FIGURES.......................................................................................................... vi
LIST OF ABBREVIATIONS.............................................................................................. viii
ACKNOWLEDGEMENTS................................................................................................. x

PART I INTRODUCTION

1.1 Overview

1.2 Terminal differentiation of cytotrophoblast and syncytiotrophoblast
   1.2.1 Syncytial fusion: its turnover and apoptosis
   1.2.2 Syncytial fusion: A membrane lipid associated event
   1.2.3 Factors mediating trophoblast syncytialization

1.3 Cell-cell interactions
   1.3.1 The cadherin superfamily
   1.3.2 Classical cadherin-catenin interactions
   1.3.3 Signaling through cadherins

1.4 Identification of the classical cadherins present in the human placenta
   and during trophoblast differentiation

1.5 Cadherin-associated transcription factors
   1.5.1 The Snail family
   1.5.2 Gene and protein structure of the human Snail (SNAI1)
   1.5.3 Gene and protein structure of the human Slug (SNAI2)
   1.5.4 The roles of human Snail and E-cadherin during tissues development
   1.5.5 Twist- A big player in cell differentiation
   1.5.6 Twist protein in morphogenesis
   1.5.7 Twist in human
   1.5.8 H-Twist gene

PART II. HYPOTHESIS AND OBJECTIVE
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Two simultaneous trophoblast differentiation pathways that occur during the first trimester of gestation</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Domain organization of representative members of the cadherin superfamily</td>
<td>16</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Cadherin/catenins complex in adherens junction</td>
<td>20</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Gene and protein structure of SNAI 1</td>
<td>29</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Gene and protein structure of SNAI 2</td>
<td>30</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Gene and protein structure of Twist</td>
<td>37</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Schematic diagram summarizing the QC-PCR strategy employed in the study of E-cadherin</td>
<td>41</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Schematic diagram summarizing the QC-PCR strategy employed in the study of Snail</td>
<td>43</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Schematic diagram summarizing the QC-PCR strategy employed in the study of Slug</td>
<td>45</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Schematic diagram summarizing the QC-PCR strategy employed in the study of Twist</td>
<td>47</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Determination of the optimal amount of internal standard E-cadherin cDNAs to be added to the QC-PCR mixtures</td>
<td>55</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Time-dependent inhibition of E-cadherin transcript expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP</td>
<td>57</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Time-dependent inhibition of E-cadherin protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP</td>
<td>61</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Immunolocalization of E-cadherin in BeWo cell cultures.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Determination of the optimal amount of internal standard Snail cDNAs to be added to the QC-PCR mixtures</td>
<td>65</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Time-dependent induction of Snail transcript expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP</td>
<td>67</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Time-dependent induction of Snail protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP</td>
<td>71</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Immunolocalization of Snail in BeWo cell cultures.</td>
<td>72</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Immunolocalization of Snail in the villous and syncytial trophoblast layers of the first-trimester placenta</td>
<td>73</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Determination of the optimal amount of internal standard Slug cDNAs to be added to the QC-PCR mixtures</td>
<td>76</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Time-dependent effects of Slug mRNA transcript expression in BeWo choriocarcinoma cells</td>
<td>78</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Time-dependent effects of Slug protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP</td>
<td>80</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Determination of the optimal amount of internal standard Twist cDNAs to be added to the QC-PCR mixtures</td>
<td>83</td>
</tr>
</tbody>
</table>
Figure 24  Time-dependent induction of *Twist* transcript expression in BeWo 85 choriocarcinoma cells by 1.5mM 8-bromo-cAMP

Figure 25  Time-dependent induction of Twist protein expression in BeWo 88 choriocarcinoma cells by 1.5mM 8-bromo-cAMP

Figure 26  Proposed signaling pathway that regulate the subcellular localization 94 of Snail in BeWo cells
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeWo</td>
<td>human choriocarcinoma cell line</td>
</tr>
<tr>
<td>CAM</td>
<td>cell-adhesion molecules</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CSF</td>
<td>colony-stimulating factor</td>
</tr>
<tr>
<td>Cyclic-AMP</td>
<td>cyclic-adenosine monophosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>E-cad</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>acceleration of gravity</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>hPL</td>
<td>human placental lactogen</td>
</tr>
<tr>
<td>HERV-W</td>
<td>human endogenous retrovirus</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Min</td>
<td>minutes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
</tbody>
</table>

viii
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZO1</td>
<td>occludin</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

My deepest gratitude and appreciation goes to my supervisors, Dr. Colin D. MacCalman and Dr. Peter C.K. Leung for their supervision and resources throughout my studies. Their advice and guidance have proven valuable and with genuine significance to my research and further career pursuits. I would like to give my sincere thanks to Dr. Catherine Pallen for her valuable suggestions and the critical reading of my thesis. Also I would like to thank my committee members, Drs. Raja Rajamahendran and Peter von Dadelszen.

I offer my appreciation to my colleagues Ellen Zhu, Alex Beristain, York Ng, Jiadi Wen for their encouragements and suggestions.

This thesis is dedicated to my family who always encourage me to follow my dreams and always be my source of strength and support. Mom, this is especially for you, I owe more than words can describe for your lifelong devotion and guidance.

All above all I want to register the thanks and acknowledge of my heart, in the words of Philippians (4:6-7).
PART 1. INTRODUCTION

1.1 Overview

The placenta is a unique, transient extraembryonic organ that permits the growth and survival of the developing embryo within the female reproductive tract. The establishment and outcome of pregnancy critically depend on intimate physical contact and functional cooperation between the trophoblast and the uterus. The trophoblast is extraembryonic fetal tissue derived from trophectoderm of the blastocyst. Trophectoderm is the earliest outermost epithelial layer and its apical interaction with the uterine luminal epithelium is a key step in implantation (Pijnenborg et al., 1980; Aplin, 1991). Implantation in humans involves the embryo invading the maternal uterine luminal epithelium to become embedded within the underlying endometrial stroma, eventually leading to the formation of a haemochorial placenta (Damsky et al., 1993; Cross et al., 1994). In humans, the principal structures of the haemochorial placenta are formed within the first three weeks of pregnancy. During the course of placental development three main trophoblast populations can be identified: cytotrophoblast stem cells and two differentiated derivative cell types: the syncytiotrophoblast and the extravillous cytotrophoblast. Early syncytiotrophoblast and cytotrophoblast can be recognized during the intrusion phase of implantation (Hertig et al., 1956; Denker, 1993). Subsequently, with progression of placentation, trophoblasts can be found forming a variety of different structures, e.g., placental villi, cell islands and anchoring villi (Enders, 1968). They all arise from villous cytotrophoblast stem cells, which either fuse to produce epithelial syncytiotrophoblast that covers placental villi, or give rise to various extravillous trophoblast populations (Figure 1).
Figure 1. **Two simultaneous trophoblast differentiation pathways that occur during the first trimester of gestation.**

A. Terminal differentiation of cytotrophoblasts to syncytiotrophoblasts that form the outer lining of the placenta.

B. Differentiation of trophoblast stem cells to extravillous trophoblasts involving initial differentiation into cytotrophoblasts (B1), followed by differentiation into extravillous trophoblasts which migrate and invade the underlying decidua (B2).
The syncytium is a polarized, secretory epithelium with an important transport function. Its formation and function are essential for the establishment and maintenance of pregnancy. Defects in its formation are suspected to lead to several complications such as preeclampsia (Lee et al., 2001) and intrauterine growth retardation (Ishihara et al., 2002) and to be compromised in Down’s syndrome (Massin et al., 2001). For these reasons, it is very important to gain a better understanding of 1) the cellular and molecular mechanisms underlying the differentiation of syncytial trophoblasts and 2) the regulation of this important biological process. To date, syncytiotrophoblast fusion has been studied in the context of a variety of biological processes, including apoptosis, membrane biochemistry, and the biology of vesicular trafficking and cell adhesion.

In this introduction, the terminal differentiation of trophoblasts will be described, with particular emphasis on synctiotrophoblast formation and function. The cellular mechanisms that are believed to modulate trophoblast differentiation will be discussed. The cell biology of the cadherin gene superfamily which has been previously detected in the human placenta and correlated with the terminal differentiation and fusion of human trophoblastic cells will be reviewed. Finally, the potential roles of several cadherin-associated transcription factors during tissue development will be discussed.

1.2 Terminal differentiation of cytrophoblast and syncytiotrophoblast

The syncytiotrophoblast of the definitive placenta is generated by continuous cell fusion of underlying cytrophoblasts and then maintained as a steady-state structure at the villous maternal-fetal interface throughout the pregnancy. The term syncytiotrophoblast was introduced about 100 years ago to designate the situation in which a multinucleate condition is
established by the fusion of initially separate cells. The basic features of the histology of the mammalian placenta were poorly understood until the important contributions of Delporte in 1912. In his study of the implantation of the human ovum, he first described the differentiation of cytotrophoblastic cells into syncytiotrophoblasts and illustrated a number of transitional stages between the syncytiotrophoblast and the cytotrophoblasts (Delporte, 1912). Alternatively, Bargmann and Knoop (1959) suggested that the syncytium was, in fact, a plasmodium of which the multinucleated syncytial trophoblast was a result of repeated nuclear division without cytokinesis.

The origin of the multinucleate trophoblast was not unraveled until functional evidence was provided by the studies of Richart (1961), examining \(^3\)H-thymidine incorporation in the trophoblastic cells of the human placenta. These and subsequent studies demonstrated that the villous cytotrophoblasts are mitotically active and that nuclear division is completely absent in the multinucleated syncytiotrophoblast in vivo.

The majority of the biological functions of the human placenta are performed by the syncytial trophoblast layer, which covers the whole placenta. It is responsible for transporting nutrients and gases from the maternal to the fetal circulation. Additionally, the multinuclear syncytiotrophoblast represents the major endocrine activity of placenta since it secretes hormones such as human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) that are essential for placental growth and for maternal adaptation to pregnancy (Hoshina et al., 1982; Benirschke and Kaufmann, 2002; Hoshina et al., 1983). This epithelial structure is also the most important maternal-fetal barrier because the terminally differentiated trophoblast is not permissive to infection by a number of viruses.
in vitro and in vivo including human immunodeficiency virus type I (McGann et al., 1994; MacCalman et al., 1996).

Progress in our understanding of human trophoblast differentiation has been limited by the fact that in vivo human experimentation is not ethically feasible and by the morphological differences that exist between the human placenta and that of experimental and domestic animals. Consequently, the trophoblastic cell lines derived from choriocarcinoma cells have provided a powerful alternative for studying trophoblast cell biology and placental development. Choriocarcinoma is a relatively rare malignant tumor of the human placenta that is comprised of mitotically active cytotrophoblasts (Benirschke & Kaufmann, 2002). To date, several choriocarcinoma cell lines have been established that exhibit varying degrees of differentiation in culture; in particular, the BeWo cell line is widely used in studying trophoblast terminal differentiation and thus will be discussed in more detail below.

1.2.1 Syncytial fusion: its turnover and apoptosis

The terminally differentiated syncytiotrophoblast has no generative potency and thus is dependent upon continuous fusion of cytotrophoblasts into the existing syncytiotrophoblast (Benirschke & Kaufmann, 2002). Continuous fusion of the villous cytotrophoblast with the syncytiotrophoblast is not only the basis for growth of the syncytiotrophoblast but also a prerequisite to keep the syncytiotrophoblast alive. Several studies have shown that loss of the villous cytotrophoblast or absence of syncytial fusion results in degeneration of the syncytiotrophoblast within a few days, as demonstrated in vitro and in animals systems (Fox, 1970; Panigel and Myers, 1972; Castellucci et al.,
1990). Others have proposed the concept that the syncytiotrophoblast maintains steady-state equilibrium at the maternal-fetal interface. In this model, the cytotrophoblast fusion provides a steady-state input to the villous syncytiotrophoblast; the output from the system is trophoblast shedding into the maternal circulation (Huppertz et al., 2001; Johansen et al., 1999; Redman and Sargent 2000). Noticeably not all villous trophoblast cells fuse syncytially; rather a pool of proliferating stem cells is preserved until term (Benirschke & Kaufmann, 2002). This suggests that syncytial fusion is tightly regulated and is restricted only to the higher differentiated stages of cytotrophoblasts underlying the syncytiotrophoblast. So what regulates this equilibrium? Apoptosis, a form of cell death which is clearly distinct from necrosis, is characterized in its end stages by nuclear chromatin condensation and cytoplasmic dehydration but with preservation of organelle integrity (Kerr et al., 1995; Erckson, 1997). Several components of the molecular machinery that regulate apoptosis are present in the villous cytotrophoblast. These include FasL and Bcl-2, in addition to the enzymes caspase-3 and transglutaminase II which appear to exist only in their inactive forms in cytotrophoblasts (Sakuragi et al., 1994; Marzioni et al., 1998; Hammer et al., 1998). The idea of apoptosis being involved in villous trophoblast turnover is further confirmed by the observation that phosphatidylserine (PS) moves from the inner plasma leaflet to the cell surface, a process known to be an early step in apoptosis (reviewed by Kagan et al., 2000). The phosphatidylserine flip into the outer plasma leaflet is likely to be initiated by activated ICE-like caspases (Renvoize et al., 1997; Black et al., 2004). Recent morphological studies of villous trophoblasts suggest that syncytiotrophoblast differentiation proceeds through two phases. On entering the syncytium, cells are committed to a long-
programmed pre-apoptotic phase. Instead of completing the entire apoptosis process, high levels of apoptosis inhibitors block the progression of the apoptosis cascade. At that time, the syncytiotrophoblast has the highest metabolic rate, as well as transport capacity. Thereafter, the apoptosis cascade is re-started and completed and the respective syncytial compartments are shed into the maternal circulation (Huppertz et al., 1998).

1.2.1 Syncytial fusion: A membrane lipid associated event

Phosphatidylserine (PS) externalization appears to be an indispensable event in the terminal differentiation and fusion of the cytotrophoblast. Differentiation and fusion of the villous cytotrophoblast result in redistribution of plasma membrane phospholipids with enrichment of PS on the syncytiotrophoblast surface (Lyden et al., 1993; Katsuragawa et al., 1995; Rote et al., 1995; Adler et al., 1995). The importance of enrichment of PS on the trophoblast cell surface during terminal differentiation is confirmed by the observation that a monoclonal antibody against a PS-dependent antigen completely blocked the intercellular fusion process in trophoblast models (Adler et al., 1995). Normally phospholipids are maintained in an asymmetrical distribution in the plasma membrane of virtually all cells with cholinephospholipids (sphingomyelin and phosphatidylcholine) located in the outer leaflet and aminophospholipids (phosphatidylethanolamine and PS) found in the inner leaflet (Daleke et al., 2000). The asymmetric distribution of phospholipids is actively maintained through the action of an Mg/ATP-dependent aminophospholipid translocase that flips PS from the cell surface to the inner leaflet (Connor et al., 1990). Phospholipid asymmetry is disrupted when PS is preferentially externalized during apoptosis (Fadok et al., 1992; Koopman et al., 1994).
or by intercellular fusion, such as when myoblasts form myotubes in muscle development (Sessions et al., 1981; Sessions et al., 1983), when sperm and oocyte membranes fuse during fertilization (Gadella et al., 2000), or when villous cytotrophoblasts form the syncytiotrophoblasts (Rote et al., 1995; Adler et al., 1995). However, the externalization of phosphatidylserine alone is not sufficient to trigger syncytial fusion. Many cells in the body become apoptotic and externalize PS without undergoing cell fusion. Syncytial fusion is an extremely specific phenomenon that only happens to cells of the same lineage. With the exception of sperm/oocyte fusion, physiological heterotypic fusion of cells is not known. Erythrocytes and the surface of the syncytiotrophoblast come into contact, both of which show externalization of PS (Connor et al., 1990), but they never fuse spontaneously. Thus, the initiation of syncytial fusion requires a more tissue-specific recognition mechanism.

1.2.3 Factors mediating trophoblast syncytialization

1.2.3a HERV-W Env Glycoprotein

The envelope (env) protein of a human endogenous retrovirus (HERV-W) called syncytin is expressed in human trophoblasts (Harris 1998; Blond et al., 2000; Mi et al., 2000). High expression levels of syncytin in human trophoblasts have suggested a role in trophoblast development for this fusogenic protein. In particular, a rabbit polyclonal antibody raised against a mixture of Env-W peptides is able to partially inhibit heterologous fusion between the choriocarcinoma BeWo cell line and COS reporter cells (Mi et al., 2000). The function of syncytin in trophoblast differentiation is further strengthened by its regulated expression during spontaneous differentiation and fusion of
primary cultures of human villous cytotrophoblasts in vitro to form syncytium (Frendo et al., 2003). The expression of HERV-W is upregulated during forskolin-induced differentiation of BeWo cells, which results in syncytium formation (Lin et al., 1999). Moreover, by using specific antisense oligonucleotides, inhibition of Env-protein expression leads to a decrease in trophoblast fusion and differentiation in vitro.

1.2.3b hCG and cAMP

Human Chorionic Gonadotropin (hCG) is produced by trophoblasts throughout the course of pregnancy (Cedard et al., 1970; Demers et al., 1973; Genti-Ramondi et al., 1981; North et al., 1990; Shi et al., 1993). hCG produced primarily by syncytiotrophoblast has intracrine, autocrine, paracrine and endocrine actions during gestation. One of its autocrine and paracrine actions is associated with the formation of the syncytiotrophoblasts by promoting the fusion of cytotrophoblasts (Cronier et al., 1994; Cronier et al., 1995; Cronier et al., 1997). In addition, exogenous hCG can also increase the morphological and functional differentiation of cytotrophoblasts in a dose- and time-dependent manner (Cronier et al., 1995).

Cyclic AMP analogues or agents which activate adenylate cyclase have profound effect upon gene expression and the morphology and function of trophoblastic cells in vitro. Although many of the effects of cAMP are at the level of gene transcription, posttranslational regulation also plays a significant role. Proteins and/or transcripts whose levels are known to be affected by cAMP in the placenta include the chorionic gonadotrophin subunit (Ringler et al., 1989), placental lactogen (Harmen et al., 1987), steroidogenic enzymes, including cytochrome P450scc, adrenodoxin, aromatase, and 17β-hydroxysteroid dehydrogenase (Picardo-Leonard et al., 1988; Lobo and Bellino,
1989; Ringler et al., 1989; Tremblay et al., 1990; Ritvos and Voutilainen, 1992). Other proteins are also reported to be affected by cAMP, including pregnancy-specific β1 glycoprotein (Kato and Braunstein, 1989), urokinase (Queenan et al., 1987) and the plasminogen activator inhibitors (Coutifaris et al., 1990). Similar to primary cultures of trophoblasts, BeWo choriocarcinoma cells undergo striking changes in response to forskolin or cAMP analogues (Wice et al., 1990). In the basal state, these cells have morphological features of intermediate trophoblasts. In response to forskolin or 8-bromo-cAMP, they show a marked reduction in DNA synthesis. Within 48 to 96h of treatment, cells begin to fuse to form large syncytia (Coutifaris et al., 1991). There is also an increase in microvilli. These morphological alterations are not seen in JEG-3 choriocarcinoma cells treated with exogenous cAMP, likely because these cells do not differentiate in response to cAMP. The stimulation of syncytium formation in BeWo cells reflects the effects of cAMP on promoting cell differentiation.

1.2.3c VEGF, CSF-1 and GM-CSF

Many growth factors have been implicated in the autocrine and paracrine regulation of cytotrophoblast function. However, much of this area of research focuses on cell motility and vascular invasion (Lash et al., 1999; Cartwright et al., 1999) and little is known about the functional role of these cytokines in trophoblast differentiation. Throughout gestation, vascular endothelial growth factor (VEGF) is expressed in term cytotrophoblasts and syncytiotrophoblast (Clark et al., 1998; Shore et al., 1997). Immunostaining has proven that high levels are present during early placentation, between gestational weeks 6 and 16, and this level decreases as pregnancy progresses (Shiraishi et al., 1996). The presence of the VEGF receptor, Flt-1, on both the
cytotrophoblast and syncytiotrophoblast has suggested a potential role for VEGF in placental angiogenesis and uterine invasion (Charnock-Jones et al., 1994). In addition to the well-established role for extravillous trophoblast in motility and invasion, VEGF has been demonstrated to enhance differentiation into syncytiotrophoblast of first-trimester cytotrophoblasts in vitro (Crocker et al., 2001).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (CSF-1) are lymphohaemopoietic cytokines originally characterized by their effects on the proliferation, differentiation, and function of myeloid haematopoietic cells (Clark and Kamen et al., 1987). These cytokines have also been implicated as pivotal mediators of placental growth and development (Pollard, 1991). In the mouse, uterine concentrations of CSF-1 increase 1000-fold in pregnancy under the control of the sex steroids, estrogen and progesterone (Bartocci et al., 1986), with a concomitant upregulation of CSF-1 receptor (CSF-1R) expression in trophoblasts (Regenstreif et al., 1989). The coordinated expression of CSF-1 and its receptor at the feto-maternal interface is temporally associated with the period of maximal placental growth (Arceci et al., 1989). In addition, the physiological relevance of these cytokines in the normal initiation and maintenance of pregnancy is further highlighted by the observation that homozygous mice genetically deficient in CSF-1 are sub-fertile (Pollard et al., 1991). In humans, serum and amniotic fluid levels of CSF-1 increase throughout gestation and CSF-1 mRNA has been detected in endometrial, decidual, and placental tissues (Ringler et al., 1989, Daiter et al., 1992, Kauma et al., 1991). The main source of pregnancy-associated human CSF-1 appears to be the luminal epithelium of the uterus, but first trimester cytotrophoblasts have also been shown to express CSF-1 (Daiter et al.,
In addition, placental stromal fibroblasts have been shown to produce GM-CSF and CSF and these cytokines are upregulated by IL-1 and TNFα (Garcia-Lloret et al., 1994), both of which are secreted by trophoblasts. CSF-1 receptor mRNA has been detected in primary cytotrophoblasts and in BeWo choriocarcinoma cells. The expression of the CSF-1 receptor increases as cytotrophoblasts fuse and differentiate to form syncytiotrophoblast (Kauma et al., 1991). The roles of these two cytokines in trophoblast differentiation are further confirmed by the observation that GM-CSF and CSF can promote the formation of multinucleated structures in primary cytotrophoblast cultures (Garcia-Lloret et al., 1994). Furthermore, CSF-1 has been shown to primarily induce hCG secretion, while GM-CSF significantly stimulates the production of hPL (Morrish et al., 1998).

1.2.3d EGF and TGF

In many species, EGF induces differentiation, proliferation, synthesis of specific proteins, and metabolic activity in various cell types (Carpenter et al., 1979). The cellular localization of EGF and EGF receptor was immunohistochemically analyzed in human placenta at different gestation stages (Takeshi et al., 1995). In 4-to 5-week old placenta, EGF and EGF receptor are almost exclusively localized to cytotrophoblasts, whereas EGF and EGF receptor are predominantly localized to syncytiotrophoblast in 6- to 12-week placenta. The staining intensity of EGF receptor in the syncytiotrophoblast is highest in the first trimester and declines toward the end of gestation. This suggests that the expression of EGF receptor is most pronounced during early placentation. The simultaneous expression of EGF and EGF receptor in cytotrophoblast and syncytiotrophoblast implies that EGF may act in an autocrine manner (Maruo and
Mochizuki, 1987; Laines-Llave et al., 1991; Maruo et al., 1992). The action of EGF on proliferation and differentiation is gestationally dependent. In cultures of very early 4-to 5-week placental explants, EGF treatment has no significant effect on the secretion of hCG and hPL to the serum-free medium throughout the entire culture period. Nevertheless, EGF enhances the proliferative activity of cytotrophoblasts in cultures of very early placental tissues obtained at 4-5 weeks gestation. However, in cultures of early 6-to 11-week placental explants, EGF treatment significantly augmented hCG and hPL secretion compared with EGF-untreated cultures, but did not affect the proliferative activity of cytotrophoblasts (Morrish et al., 1987). Some groups have also demonstrated that EGF changes the morphology of the primary cytotrophoblasts, inducing a marked increase in syncytiotrophoblast formation without proliferation (Morrish et al., 1987). The evidence that EGF is involved in trophoblast differentiation is further strengthened by experimental results from the use of an anti-EGF neutralizing monoclonal antibody that acts as an antagonist of the EGF/EGF-receptor. The neutralizing monoclonal antibody exhibits a significant inhibitory effect on the secretion of both hCG and hPL, demonstrating a clear role of EGF during trophoblast differentiation (Amemiya et al., 1994).

Transforming growth factor β1 is a multifunctional growth factor present in many cells that can have both positive and negative effects on cell proliferation and differentiation (Sporn et al., 1986; Massague et al., 1987). TGFβ1 induces differentiation of keratinocytes (Shipley et al., 1986; Mansridge et al., 1988; Coffey et al., 1988); ovarian thecal cells (Skinner et al., 1987) and bronchial epithelium (Masui et al., 1986). It can also inhibit differentiation in other cells, including myoblasts (Florini et al., 1988),
adipocytes (Ignotz and Massague, 1985), and early hematopoietic progenitor cells (Ohta et al., 1987). Interestingly, TGFβ1 can act both as an inducer, and an inhibitor of differentiation in cartilage (Frenz et al., 1990). In human placenta, mRNAs encoding isoforms of TGFβ (β1, β2, β3) have been detected in syncytiotrophoblasts (Ando et al., 1998; Caniggia et al., 1999). In addition, both type I and type II TGFβ receptors have been identified in trophoblasts (Ando et al., 1998). These findings suggest that TGFβ may act as an autocrine/paracrine factor to regulate placental development. Notably, TGFβ1 appears to play an inhibitory role in syncytiotrophoblast formation (Morrish et al., 1991). TGFβ1 not only inhibits morphological syncytial formation from cytotrophoblasts but also inhibits the expression levels of both hCG and hPL in vitro. TGFβ1 is also reported to decrease the estradiol and progesterone production in the JEG-3 choriocarcinoma cell line.

1.3 Cell-cell interactions

Freshly isolated mononucleate cytotrophoblasts aggregate and establish extensive interactions with one another through the formation of desmosomes, adherens and gap junctions. The cytotrophoblasts eventually undergo terminal differentiation and spontaneously fuse into multinucleated syncytium. It is well accepted that morphogenesis and cell differentiation depend in part on the regulated expression of cell surface proteins, which, through their connections to the cytoskeleton, result in altered gene expression (Edelman, 1998). In light of that theory, there is likely to be tightly regulated expression in trophoblasts of members of the cadherin gene superfamily of calcium-dependent cell
adhesion molecules (CAM), as the cadherins are believed to be fundamental players in this morphologic and functional differentiation.

1.3.1 The cadherin superfamily

Cadherins are a large family of glycoproteins that mediate calcium-dependent cell adhesion in a homophilic manner. Members of this gene family are comprised of an extracellular domain responsible for cell-cell interactions, a transmembrane domain, and a cytoplasmic domain that is linked to the cytoskeleton. Cadherins are also characterized by a distinctive sequence motif termed a cadherin repeat which is tandemly repeated in their extracellular domain (reviewed by Yagi & Takeichi, 2000). Structural studies have shown that calcium ions bind to each cadherin repeat to ensure proper folding and protect against protease digestion, thus conferring rigidity upon the extracellular domain (Yoshida & Takeichi, 1982; Koch et al., 1999).

The cadherin family of proteins consists of classical and non-classical cadherins (Figure 2). The classical cadherins are the main mediators of calcium-dependent cell-cell adhesion (reviewed by Yagi & Takeichi, 2000). The non-classical cadherins include desmosomal cadherins (Angst et al., 2001), the recently discovered large subfamily of protocadherins which are implicated in neuronal plasticity, and the seven-transmembrane cadherins or Flamingo cadherins (Nollet et al., 2000) which were first identified in Drosophila. Flamingo cadherins are believed to mediate planar cell polarity in Drosophila (Chae et al., 1999).
Figure 2. Domain organization of representative members of the cadherin superfamily. Classical cadherins have a well conserved cytoplasmic domain and can be grouped into Type I and Type II cadherins, with Type I cadherins having a His-Ala-Val sequence in the N-terminal region. The desmosomal cadherins contain five extracellular CRs and a cytoplasmic domain that interacts with plakoglobin, desmoplakin and the plakophilins. The proto-oncogene RET has one CR and a tyrosine kinase domain in its cytoplasmic domain. Flamingo cadherins have 8-9 CRs, 2 laminin AG domains, 4 EFG domains, and a unique domain called the Flamingo box.
Classical cadherins are a family of single-span transmembrane glycoproteins and are further categorized into 2 types, Type I and Type II. The Type I classical cadherins include three that were originally identified by immunological and cDNA cloning strategies: E-cadherin (E-cad), N-cadherin (N-cad) and P-cadherin (P-cad) (Nose and Takeichi, 1986; Nagafuchi et al., 1987; Hatta et al., 1988; Suzuki et al., 1991). These cadherin subtypes are the best characterized members of this gene superfamily of CAMs and were named on the basis of their tissue distribution during mouse embryonic development; E-cad is primarily expressed in epithelial cells, N-cad in neuronal cells and P-cad in the placenta and deciduas. Type I and Type II cadherins both have five extracellular repeats, and Type I can be distinguished from Type II by the presence of a histidine, alanine, valine (HAV) tripeptide within the most N-terminal extracellular repeat (EC1). Homophilic protein-protein interactions between two cadherin molecules at the cell surface are believed to be mediated by interactions between the HAV domains. Cadherin molecules with a deletion in EC1 fail to mediate cell-cell interactions (Takeichi, 1990; Knudsen et al., 1998). In addition, recent crystallization of the extracellular domain of the Type I cadherin, *Xenopus* C-cadherin, further identified other intracellular interfaces participating in cadherin interactions, such as the conserved tryptophan side chain that intercalates into a conserved hydrophobic pocket in the corresponding partner (Boggon et al., 2002). These CAMs are synthesized as precursor molecules containing a signal peptide and an amino terminal domain that is removed post-translationally by the furin/subtilisin family of proprotein convertases (Ozawa and Kemler, 1990; Posthaus et al., 1998). The extracellular domain of the cadherins is also glycosylated on asparagine residues prior to being transported to the cell surface (Shore and Nelson, 1991; Geyer et
In addition, the EC5 subdomain of the Type I classical cadherins contain four conserved cysteine residues that are likely involved in the formation of intramolecular disulfide bonds (Ringwald et al., 1987, Takeichi, 1991). The cytoplasmic domains are the most highly conserved regions among members of the Type I classical cadherin gene superfamily (Hatta et al., 1988; Suzuki et al., 1991). Recent studies have implicated these cytoplasmic domains in regulating cadherin function at the cell surface (Ozawa and Kemler, 1998; Yap et al., 1998). The classical cadherin cytoplasmic domains are capable of interacting with a group of cytoplasmic proteins, known as the catenins.

1.3.2 Classical cadherin-catenin interactions

At the adherens junctions, the intracellular domain of the classical cadherins interacts with various catenin proteins to form the cytoplasmic cell-adhesion complex (CCC) (Figure 3). β-catenin and γ-catenin, also known as plakoglobin, bind in a mutually exclusive way to the same conserved site comprising a core region of 30 amino acids at the carboxyl terminal of classical cadherins (Stappert et al., Jou et al., 1995). β-catenin and γ-catenin are both members of the Armadillo family of proteins and share about 65% identity (Fouquet et al., 1992). Apparently these two proteins play very different roles in cellular signaling pathways (Zhurinsky et al., 2000), however they can substitute for one another as structural components of the adherens junction (Butz et al., 1992; Knuden et al., 1992). The N-terminal portion of both β- and γ- catenins interacts with α-catenin, which links the cadherin to the cytoskeleton. α-catenin is crucial in maintaining the integrity of adherens junctions since knocking-down or mutation of α-catenin disrupts adherens junctions (Aberle et al., 1994; Jou et al., 1995; Pokutta & Weis, 2000;
Nagafuchi & Tsukita, 1994). Further studies showed that both the N-terminus and C-terminus of α-catenin are needed to connect the cadherin complex to the cytoskeleton (Nagafuchi et al., 1994). Several actin-binding proteins such as α-actinin, vinculin and ZO1 are responsible for linking α-catenin to the actin cytoskeleton, although in some cases α-catenin can interact with the actin cytoskeleton directly (Knudsen et al., 1995; Watabe-Uchida et al., 1998).

In addition to β-catenin and plakoglobin, another catenin called p120 catenin binds to the cytoplasmic domain of Type I and Type II classical cadherins. p120 catenin is a member of a subgroup of Armadillo family members that have been shown to be localized in various cellular junctions (Anastasiadis & Reynolds, 2000). p120 catenin was initially identified as Src substrate and subsequently shown to interact with the highly conserved juxtamembrane domain of cadherins (Reynolds et al., 1994). The role played by p120 in adherens junctions is still elusive, probably because there are numerous p120 catenin splice variants and a number of closely related family members that hinder detailed investigation of each kind. In addition to their roles in regulating cadherin-mediated adhesion and linkage to the cytoskeleton, the catenins have been shown to be integral parts of signal transduction pathways.
Figure 3. Cadherin-cadherin and cadherin-catenin complexes at the adherens junction. Trans-interactions between two cadherins molecules are mediated by homophilic protein-protein interactions. These, and cadherin-catenin interactions, form molecular complexes that are linked to the actin cytoskeleton and regulate intracellular signaling pathways.
1.3.3 Signaling through cadherins

Wnt

At the adherens junctions, $\beta$-catenin is well known as a participant in mediating cadherin-based, cell-cell adhesion. On the other hand, $\beta$-catenin is also an integral part of the Wnt signaling pathway involved in both development and tumorigenesis. Wnt is an extracellular matrix-associated growth factor that interacts with its receptor, a member of the frizzled family, to initiate a signal transduction pathway that ultimately promotes cell growth by inducing cyclin D1 and myc (Conacci-Sorrell et al., 2002). In the absence of Wnt signaling, a pool of non-adhesion-associated, soluble $\beta$-catenin binds to the adenomatous polyposis coli (APC)-axin tumor suppressor complex, where it is phosphorylated by glycogen synthase kinase 3$\beta$ and targeted for degradation by ubiquitin. In contrast, in response to Wnt signaling, $\beta$-catenin degradation is inhibited, allowing it to accumulate and move to the nucleus where it forms a complex with the TCF/LEF transcription factors to drive transcription of Wnt responsive genes. There is strong evidence that the expression of E-cadherin can modulate the activity of $\beta$-catenin-associated Wnt signaling. This idea comes from transfection studies where overexpression of E-cadherin can retain $\beta$-catenin/TCF signaling (Gottardi et al., 2001).

MAPK

Regulated E-cadherin expression is believed be compromised in abnormal cell growth and differentiation. Increasing evidence has shown that disruption of E-cadherin expression and function not only has an effect on the cell-cell adhesion system, but also on intracellular signal transduction. The kinase cascade regulating the activity of p42/p44
mitogen-activated protein kinases (MAPK) is one of the best characterized intracellular signaling pathways, and it integrates signals coming from a wide array of cell membrane receptors (Seger et al., 1995). The MAPK pathway has been found to play a critical role in the control of cell survival, proliferation, and differentiation when activated by both growth factor receptor and surface adhesion molecules such as integrins, in a variety of cellular settings (Howe et al., 1998; Zhu et al., 1999). A recent report by Gutkind et al. (2000) has demonstrated that E-cadherin is capable of activating MAPK in epithelial cells. Furthermore, E-cadherin is able to transactivate EGFR in a ligand-independent manner, although the mechanism is still unknown. The use of tyrphostin AG 1478, which specifically inhibits EGFR kinase by competing for ATP, decreases MAPK activation induced by the formation of adherens junctions after calcium restoration. The current idea is that E-cadherin and EGFR cooperate to activate signaling pathways downstream of adherens junctions, thus leading to the phosphorylation of Shc and an increase in the enzymatic activity of MAPK (Gutkind et al., 2000).

1.4 Identification of the classical cadherins present in the human placenta and during trophoblast differentiation

Cadherin superfamily members not only maintain the structural integrity of cells and tissues but also control a wide array of cellular behaviors (Larue et al., 1996; Huber et al., 1996). They are key regulators of cell and tissue polarization (Larue et al., 1994; Riethmacher et al., 1995), and they also regulate cell movement such as cell sorting, cell migration and cell rearrangements (Nose et al., 1988; Steinberg & Takeichi, 1994; Takeichi, 1998). The importance of cadherins is evidenced by the embryonic lethality or
abnormal tissue maturation in mice null for specific cadherins (Hynes, 1996). In view of
the central role that the classical cadherins play in the formation and organization of
tissues during embryonic development and in maintaining tissue integrity, it is not
surprising that members of this gene superfamily mediate human placentation.

The expression of E-cadherin during morphologic differentiation of trophoblastic
cells has been well studied (Coutifaris et al., 1991). Unlike the cytotrophoblasts isolated
from human chorionic villi that aggregate and fuse spontaneously to form syncytium in
vitro, JEG-3 and BeWo choriocarcinoma cells are not fusion competent under standard
culture conditions. During the aggregation of primary cytotrophoblasts, E-cadherin is
localized on the cell surface at points of cell-cell contact and cannot be detected
following cellular fusion. E-cadherin protein levels in normal cytotrophoblasts increase
during the initial 24h of culture, but decrease by 82% between 24h and 96h of culture,
coincident with a marked decrease in the number of cellular aggregates and a parallel
increase in the formation of multinucleated syncytia. The disappearance of E-cadherin
from the cell surface was also observed in BeWo choriocarcinoma cells undergoing
terminal differentiation and fusion in response to the intracellular second messenger,
cAMP (Coutifaris et al., 1991).

In contrast to E-cadherin, the expression of Cadherin-11 increases during the
formation of multinucleated syncytium in primary cultures of villous trophoblasts.
Antisense oligonucleotides specific for Cadherin-11 are able to inhibit the terminal
differentiation and fusion of primary villous cytotrophoblasts (MacCalman, 1997). The
fusogenic ability of Cadherin-11 was further confirmed by the exogenous expression of
Cadherin-11 in mononucleate JEG-3 cells, which are not fusion competent. The
exogenous expression of Cadherin-11 is capable of promoting the morphological and functional differentiation of these trophoblastic cells with a concomitant decrease of E-cad and increase of hCGβ mRNA levels (Getsios et al., 2003). Collectively, these results indicate that E-cadherin and Cadherin-11 are differentially expressed during trophoblast differentiation in vitro and are key players mediating the differentiation process.

1.5 Cadherin-associated transcription factors

Cloning and characterization of the human E-cadherin promoter has revealed that two E-boxes present in a proximal fragment of the promoter are important for the repression of this gene (Giroldi et al., 1997). The two E-boxes characterized so far in the human and mouse E-cadherin promoters have an identical core, consisting of the sequence 5′CACCTG. This sequence exactly matches the DNA-binding site of Snail (Mauhin et al., 1993; Battle et al., 2000). Snail is an important regulator of E-cadherin gene expression in several developmental processes in different species, from flies to humans (reviewed by Hamavathy et al., 2000). Drosophila Snail mutant embryos die at gastrulation and show a gastrulation-defective phenotype that has been associated with impaired downregulation of E-cadherin (Oda et al., 1998). In light of the regulation of Snail on E-cadherin gene expression, Snail and its family members are believed to be fundamental players in E-cadherin-mediated trophoblast differentiation and fusion. In the next section, the cell biology of the Snail family will be discussed.

1.5.1 The Snail family

Snail was first identified in Drosophila melanogaster (Grau et al., 1984; Nusslein-Volhard et al., 1984), and has been implicated in several important embryonic
developmental processes, including mesoderm formation (Leptin, 1991; Alberga et al., 1991) neural crest formation (Nieto et al., 1994), and left-right asymmetry (Issac et al., 1997). Embryos which are homozygous for loss-of-function of Snail exhibit defects in the invagination of the presumptive mesoderm and retraction of the germ band (Grau et al., 1984; Nusslein-Volhard et al., 1984). The elucidation of the significant role of Snail in Drosophila embryogenesis prompted a search for similar genes in other organisms. Since then, homologs of Snail have been identified in diverse groups of animals including nematodes (Metzstein & Horwitz, 1999), protochordates (Corbo et al., 1997), amphibians, fish (Smith et al., 2000), and mammals (Twigg & Wilkie, 1999).

Snail family members encode zinc-finger type transcription factors (Mauhin et al., 1993). They all share a similar organization, being composed of a highly conserved carboxyl-terminal region that contains from four to six zinc fingers of which probably a minimum of four fingers are required for function. The amino-terminal region of Snail family proteins is more divergent among all members of the family.

Zinc fingers are one of the most abundant structural motifs. Different classes of zinc fingers include CCHH, CCHC, CCCH, CCCC, where C and H are the cysteine and histidine residues that constitute the zinc-binding structure. The fingers of the Snail family are all the CCHH type and function as sequence-specific DNA-binding motifs. The fingers are structurally composed of two β-strands followed by an α-helix, the amino-terminal part of which binds to the major groove of the DNA. Both random selection and transfection assays with different promoters have shown that the consensus binding site for Snail-related genes contains a core of six bases, CAGGTG (Battle et al., 2000). This motif is identical to the E-box, which is also the consensus binding site of
basic helix-loop-helix (bHLH) transcription factors, suggesting that *Snail* proteins might compete with these for the same binding sequences (Kataoka et al., 2000). On binding to the E box, *Snail* family members have been demonstrated to act as transcriptional repressors. The repressor activity depends not only on the finger region, but also on at least two different motifs that are found in the amino-terminal region. One of these is the SNAG domain. This domain was originally identified in the Gfi-1 oncoprotein (Grimes et al., 1996), which is also a zinc-finger protein, and was believed to function as a repressor domain. The SNAG domain is conserved in all vertebrate *Snail* genes, and is important for repression in mammalian cells (Nakayama et al., 1998). However, SNAG is not the only the motif responsible for repression. In *Drosophila*, there is another motif called the CtBP binding site, which interacts with a co-repressor, CtBP (carboxy-terminal binding protein) and thus as a repressor domain for *Snail* (Nibu, 1998).

### 1.5.2 Gene and protein structure of the human *Snail* gene (*SNAI1*)

Paznekas et al. (1999) mapped the human *Snail* gene to chromosome 20 using somatic cell and radiation hybrid mapping panels. It is composed of three exons with 2 intervening introns (Figure 4). The human *Snail* gene is 60.6 % identical to the coding region of human *Slug*. The structure of human *Snail* is similar to that of the mouse *Snail* gene with 85% nucleotide identity. The human and mouse *Snail* 5'UTR share 55.7% identity, while the 3'UTRs share 63% identity. The *Snail* protein is composed of 264 amino acids with a predicted molecular mass of 29.1 kDa. It is composed of 264 amino acids. Its C-terminal portion has three classic zinc fingers and a fourth atypical zinc
finger. The N-terminal portion differs much more among the various family members and contains a SNAG domain (Pazekas et al., 1999).

The human Snail protein is expressed in many tissues. By Northern blot analysis, the human Snail transcript is 2.0kb in size with no observed alternative spliceoforms. It is highly expressed in adult heart and lung, and in placenta. It is expressed at lower levels in adult brain, liver and skeletal muscle (Pazekas et al., 1999).

1.5.3 Gene and protein structure of the human Slug (SNAI 2)

In addition to Snail genes, all vertebrates contain a related Slug gene. The Slug proteins from different vertebrates constitute a distinct subgroup and they are particularly highly conserved among themselves. The human Slug gene has been mapped to chromosome 8 (Cohen et al., 1998). Similar to human Snail, it contains three exons and 2 introns (Figure 5). The Slug transcript is 2.2kb and is highly expressed in placenta and adult heart but is barely detectable in adult brain and lung. The protein is composed of 268 amino acids, with an expected molecular mass of 29.989 kDa. The human Slug protein is 95, 93 and 88% homologous to mouse, chicken and Xenopus Slug, respectively. The zinc finger regions are 100% identical between human and mouse Slug, indicating that Slug is very resistant to evolutionary changes (Cohen et al., 1998).

The expression and function of both Snail and Slug in vertebrates are still not well understood. Slug seems to be the dominantly expressed protein of the pair in chick embryos and is responsible for neural crest migration and mesoderm delamination (Nieto et al., 1994). This idea comes from the observation that antisense oligonucleotides to Slug inhibited both neural crest migration and mesoderm delamination in early chick
development (Nieto et al., 1999). Similar results were obtained from *Xenopus* when antisense oligonucleotides to *Slug* were injected into *Xenopus* embryos, as this disrupted neural crest migration and formation of specific crest derivatives (Carl et al., 1999). However, *Snail* in the chick embryo was found to be involved in left-right asymmetry (Issac et al., 1997), in contrast to its action in *Xenopus*, where *Slug* and *Snail* act redundantly to control neural crest cell migration (Carl et al., 1999).

Human *Slug* has been demonstrated to be an anti-apoptotic factor mediating the oncogenic effect of the E2A-HLF fusion protein in leukemia (Inukai et al., 1999). Interestingly, *Slug* mRNA is present in most adult tissues including spleen and thymus, but not in peripheral blood leukocytes. It has been suggested that *Slug* is present at earlier developmental stages of lymphocytes, when its anti-apoptotic function is required for positive selection (Hemavathy et al., 2000).
Figure 4. Gene and protein structure of the Snail family member SNAI1. A. The gene encoding SNAI1 comprises three exons and two introns. The green boxes represent the exons and the solid lines denote the untranslated area and intronic regions. B. The SNAI1 protein has 264 amino acids with 4 zinc fingers and a SNAG domain. Snail family members all share a similar organization, being composed of a highly conserved carboxyl-terminal region that contains from four to six zinc fingers of which probably a minimum of four fingers are required for function. The amino-terminal part of the Snail family proteins is more divergent among all members of the family.
Figure 5. Gene and protein structure of the Snail family member SNAI 2. A. The SNAI 2 gene encoding Slug comprises three exons and two introns. The green boxes represent the exons and the solid lines denote the untranslated area and intronic regions. B. The SNAI 2 protein has 268 amino acids with 5 zinc fingers and a SNAG domain.
1.5.4 The roles of hSnail and E-cadherin during tissue development

The importance of hSnail in triggering epithelial-mesenchymal transition (EMT) has been well established (Cano, 2000). EMT is the mechanism by which epithelial cells that are generated in a particular region can dissociate from the epithelium and migrate to reach different locations (Hay et al., 1995; Boyer et al., 2000). As such, EMT is fundamental to both normal development and the progression of malignant epithelial tumors. The involvement of Snail in EMT has been demonstrated by the observation that Snail can convert normal epithelial cells into mesenchymal cells through the direct repression of E-cadherin expression (Carver et al., 2001). The inverse correlation of E-cadherin and Snail expression has been seen not only during EMT but in an EMT-like process, cancer progression (Battle et al., 2000). Epithelial-mesenchymal transition is one of the components of the progression of carcinomas, particularly those having an invasive phenotype. Both EMT and progression of cancer occur concomitantly with the cellular acquisition of migratory properties following downregulation of expression of E-cadherin. The strong inverse correlation between Snail and E-cadherin expression has been seen in different carcinoma cell lines, including breast (Hajra et al., 2002), gastric (Rosivatz et al., 2002), and hepatocellular carcinoma (Sugimachi et al., 2003). Ectopic expression of Snail is able to downregulate E-cadherin in human hepatocellular carcinoma (HHC), while expression of Slug, the other Snail family member, is not related to E-cadherin expression in HHC. Recently, Hajra et al. (2000) reported that Slug is capable of repressing E-cadherin in breast cancer cell lines. They also demonstrated repression of E-cadherin by Slug and Snail in vivo (Hajra et al., 2002). Although Snail
and Slug are from the same family, their functions differ among various species and cell type.

As Snail is involved in the loss of E-cadherin expression during development and cancer progression, several groups have provided different mechanisms by which Snail or Slug mediate the transcriptional repression of E-cadherin. Phosphorylation and subcellular location both regulate the activity of Snail. The nuclear export sequence (NES) located within the N-terminal of Snail is proven to participate in the transport of Snail out of the nucleus. The activity of the NES is regulated by phosphorylation, although the kinase and the transporter that are respectively responsible for the phosphorylation and export are unknown. When Snail is phosphorylated, it is transported out of the nucleus even though the DNA binding ability is retained, and is thus not able to access its target promoters. Therefore, Dominguez et al. (2003) suggest that Snail promotes its effects only when it is upregulated and the entire population cannot be totally phosphorylated and exported. Hence some Snail is retained in the nucleus and subsequently binds to its target genes.

Epigenetic gene regulation has emerged as a main mechanism to control gene expression. This involves chromatin remodeling and histone modifications that change the conformation of the DNA. Epigenetic processes that involve hyperacetylation of histone are a common way to control gene expression, especially developmentally regulated gene expression, for example of the globin gene. Hyperacetylation of histone H3 and H4 are generally associated with transcriptionally active chromatin, while the chromatin of inactive regions is enriched with deacetylated histones H3 and H4. The acetylation status of histones at specific DNA regulatory sequences depends on the
recruitment of histone acetyltransferases or histone deacetylase (HDAC). These do not only function alone, but also recruit different co-activators or co-repressors. In Peinado et al. (2004), Snail interacts directly with the endogenous E-cadherin promoter, as demonstrated by chromatin immunoprecipitation (ChIP) assays. In addition, Snail recruits histone deacetylase (HDAC). Moreover, association of Snail with the co-repressor mSin3A is required for down-regulation of E-cadherin gene expression. These interactions are dependent on the SNAG domain in the N-terminal region of Snail.

1.5.5 Twist-A big player in cell differentiation

The basic helix-loop-helix transcription factor Twist was originally identified in Drosophila, in which mutated Twist results in a Twisted phenotype in embryos (Thisse et al., 1988). Thereafter several groups demonstrated that the expression pattern of Twist is related to the formation and specification of the mesoderm in Drosophila (Leptin, 1991). Recently, Twist was demonstrated to play a major role in inducing cell movement and tissue reorganization during invasion and metastasis (Yang et al., 2004), in particular in the metastasis of breast cancer. In addition to cancer differentiation, Twist is able to regulate osteoblast differentiation. As Twist has a regulatory role both in cancer differentiation and normal differentiation, we speculate that Twist may be regulated during trophoblast differentiation. In the next section, the role of Twist during morphogenesis and embryogenesis will be reviewed.
1.5.6 *Twist* protein in morphogenesis

During gastrulation, the mesoderm and the anterior endoderm are formed by the invagination of the ventral furrow. Several zygotic genes, under control of different maternal morphogens, set up the primordial of the germ layers. The nuclear gradient of the maternal gene product *dorsal* directs the ventral expression of two zygotic transcription factors, *Twist* and *Snail* (Belay *et al.*, 1987; Thisse *et al.*, 1988, 1991; Ip *et al.*, 1992). Both *Twist* and *Snail* are required for the formation of the ventral furrow and later, of the mesodermal germ (Simpson, 1983; Grau *et al.*, 1984). They are thought to activate the genes responsible for ventral furrow formation and for mesodermal differentiation (Leptin, 1991).

Following gastrulation, a segmentally repeated pattern of *Twist* expression forms along the anterior-posterior axis of the embryo, subdividing each mesodermal segment into a low and high *Twist* domain (Azpiazu *et al.*, 1996; Riechmann *et al.*, 1997). Cells located in the high *Twist* domain develop into somatic muscle and heart, whereas cells located in the low *Twist* domain differentiate into visceral muscle, fat body and gonadal mesoderm. High *Twist* levels direct cells to differentiate into body muscle, and low levels permit the execution of other tissue fates (Baylies and Bate, 1996).

1.5.7 *Twist* in human

In humans, mutations in the *Twist* gene have been identified in Saethre-Chotzen syndrome, a bone disease characterized by premature fusion of the cranial sutures and limb abnormalities of variable severity (El Ghouzzi *et al.*, 1997). Insertions, deletions, nonsense and missense mutations have been identified in *H-Twist*, and these gene
alterations result in protein degradation and subcellular mislocalization of the *Twist* protein (El Ghouzzi *et al.*, 2000). *Twist* has been demonstrated to alter osteoblastic differentiation patterns in bone development. *Twist* overexpressing cells are more progenitor-like, whereas cells treated with *Twist* antisense oligonucleotides and thus having reduced *Twist* expression represent a more differentiated osteoblast phenotype (Lee *et al.*, 1999). In addition, it has been shown that *Twist* plays a crucial role in cranial osteogenesis, and *Twist* haploinsufficiency enhances apoptosis in osteoblasts leading to premature fusion of calvarial bones (Yousfi *et al.*, 2002). It has been proposed that many craniosynostosis syndromes result from local perturbation of apoptotic programs that are essential for proper timing of suture fusion. (Bourez *et al.*, 1997) Recently, the role of *Twist* in apoptosis has been further elucidated. *Twist* may function as a potential oncogene interfering with p53-related pathways, leading to the prevention of myc-induced apoptosis in mouse embryonic fibroblasts (Maestro *et al.*, 1999). *Twist* is also involved in the suppression of differentiation and protection of apoptosis through inhibition of p21 via both p53-dependent and -independent pathways. *Twist* exerts its anti-apoptotic function by specifically suppressing NF-kB-dependent cytokine expression (Sosic *et al.*, 2003).

### 1.5.8 *H-Twist* gene

The human *Twist* gene is located at 7p21 (Bourgeois *et al.*, 1996). The gene encoding *H-Twist* comprises two exons separated by an intron (Figure 6). The human *H-twist* cDNA isolated from a placental cDNA library encodes a protein of 202 amino acids with a basic helix-loop-helix domain (Bourgeois *et al.*, 1996). It is 96.6% identical to the
M-Twist protein of *Mus musculus*. Overall sequence conservation is obvious among the 40 amino acids lying in the amino-end, and in the carboxy terminal moiety of these proteins, including the basic helix-loop-helix (bHLH) structure.

*Twist* is a bHLH transcription factor. The two amphipathic helices are separated by a loop with varying length, which is important in maintaining the tertiary structure of the HLH moiety. BHLH proteins bind as dimers to the consensus hexanucleotide sequence E-box, 5'CANNTG-3'. The basic domain mediates the interaction with DNA (Yin *et al.*, 1997; Cripps & Olson, 1998). Class B bHLH proteins to which *H-Twist* belongs are known to form stable heterodimers with members of class A bHLH transcription factors, including gene products of E2A (E12 and E47) (Staudinger *et al.*, 1993). In humans, E12 appears as a putative partner *in vivo* that regulates *H-twist* transcriptional activity and determines the tissue-specificity of *Twist* (El Ghouzzi *et al.*, 2000).
Figure 7. Gene and protein structure of the human *Twist*. A. The gene encoding *H-Twist* comprises two exons separated by a unique intron. B. The *H-Twist* protein has 202 amino acids with a basic helix-loop-helix domain.
PART II. HYPOTHESIS AND OBJECTIVE

A tight regulation of cadherin expression is fundamental for the differentiation of cytotrophoblasts. As demonstrated from previous studies, the cadherin subtype switch from E-cadherin to Cadherin-11 is associated with cytotrophoblast differentiation. Many transcriptional regulators and signaling molecules have been identified as playing key roles in various developmental processes. Among the transcription regulators, the Snail family and the bHLH Twist transcription factor both have significant roles in embryogenesis and tissue differentiation. In particular, a number of reports indicated that Snail or Slug is a potential regulator of E-cadherin expression. In addition, Twist is implicated in cadherin switching during tumor metastasis. Since cytotrophoblast differentiation is at least in part mediated by cadherin subtype switching, the present study aimed to elucidate the molecular mechanism(s) underlying the differential expression of cadherin molecules by examining the expression profiles of Snail, Slug and Twist during cAMP-induced differentiation of BeWo choriocarcinoma cells.
PART III. MATERIAL AND METHODS

3.1 Cell preparation and culture

A human BeWo choriocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained as a monolayer in culture flasks and induced to differentiate in vitro as described previously (MacCalman et al., 1996). Briefly, culture medium F12 (Sigma, St Louis, MO) was supplemented with 10% fetal bovine serum (Life Technologies, Inc. Burlington, ON, Canada) and penicillin and streptomycin (100IU/ml and 100ug/ml, respectively. Cultures of the BeWo choriocarcinoma cell line were treated with or without 8-bromo-cAMP (1.5 mM; Sigma-Aldrich, Canada) for 0, 12, 24, or 48 h before being harvested for total RNA extraction, protein extraction or immunostaining. For RNA extraction and Western blot analysis, BeWo cells were cultured in 35 mm² culture dishes at densities of 4X10^5 cells per dish. For immunocytochemistry, the cells were seeded on glass coverslips that had been placed in 6 well-plates at densities of 1X10^5 cells per dish. Cells were grown in a humidified incubator with 5% carbon dioxide at 37°C.

3.2 Primer design

Nucleotide sequences specific for E-cadherin, Snail, Slug, Twist were identified in the human mRNA sequences deposited in GenBank (National Center for Biotechnology Information, Bethesda, MD). Forward and reverse primers corresponding to these DNA sequences and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used to quantify and assess the integrity of the total RNA samples, and were synthesized at the Nucleic Acid Protein Synthesis Unit, University of
British Columbia. To construct internal standard cDNA fragments, floating primers with a sequence complementary to a short nucleotide sequence present in the expected PCR products were generated by attaching the complementary sequences of these binding sites to the 3' end of the original reverse primers specific for E-cadherin, Snail, Slug and Twist (Figures 7-10).

3.3 Total RNA preparation and RT-PCR

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

PCR was performed using template cDNA generated from total RNA extracts from BeWo cultures and the primer sets specific for E-cadherin, Snail, Slug or Twist. The PCR conditions were as follows: 1 min at 94°C, 1min at 58.5°C, 59.5°C or 58°C for E-cadherin, Snail, Slug or Twist, respectively, and 1.5 min at 72°C, followed by a final extension at 72°C for 15 min. Thirty PCR cycles were carried out for E-cadherin, Snail, Slug or Twist. The resultant PCR products for E-cadherin, Snail, Slug and Twist were resolved by gel electrophoresis and visualized by ethidium bromide staining. Aliquots of the E-cadherin, Snail, and Slug and Twist PCR products were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and selected clones were subjected to DNA
FIGURE 7. Schematic diagram of oligonucleotide primers for human *E-cadherin* amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers 5'-3'</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human E-cad</td>
<td>Forward Primer: TGGATGTGCTGGATGTGAAT</td>
<td>560bp</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: ACCCACCTCTAAGGCCATCT</td>
<td></td>
</tr>
<tr>
<td>Mutant Forward Primer</td>
<td>TGGATGTGCTGGATGTGAATGCTACAGACA3'</td>
<td>305bp</td>
</tr>
</tbody>
</table>

560 bp target cDNA

↑

Forward Primer: TGGATGTGCTGGATGTGAAT

3' ACCCACCTCTAAGGCCATCT Reverse Primer

5' TGGATGTGCTGGATGTGAAT

GCTACAGACA ACCCACCTCTAAGGCCATCT

305bp competitor cDNA

Mutant Forward Primer: TGGATGTGCTGGATGTGAAT GCTACAGACA ACCCACCTCTAAGGCCATCT

↓
Figure 7. Schematic diagram summarizing the QC-PCR strategy employed in the study of *E-cadherin*. A competitive E-cadherin cDNA (305 bp) was generated through the addition of a stretch nucleotides, corresponding to a specific sequence within the target cDNA (560 bp), to the initial forward primer.
FIGURE 8. Schematic diagram of oligonucleotide primers for human *Snail* mRNA amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers 5'-3'</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human <em>Snail</em></td>
<td>Forward Primer 1. GGTTCTTCTGCCTACTGCT</td>
<td>552</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer 2. CCAAGGTAGGTATTCTTG</td>
<td>552</td>
</tr>
<tr>
<td></td>
<td>Mutant Forward Primer 3. GGTTCTTCTGCCTACTGCT CCAATGCTCA</td>
<td>351</td>
</tr>
</tbody>
</table>

552bp target cDNA

Forward Primer GGTTCTTCTGCCTACTGCT

3' CCAATGCTCA CCAGGCTGAGGTATTCTTG Reverse Primer

Mutant Forward Primer GGTTCTTCTGCCTACTGCT CCAATGCTCA

351bp competitor cDNA
Figure 8. Schematic diagram summarizing the QC-PCR strategy employed in the study of Snail. A competitive Snail cDNA (351 bp) was generated through the addition of a stretch nucleotides, corresponding to a specific sequence within the target cDNA (550 bp), to the initial forward primer.
FIGURE 9. Schematic diagram of oligonucleotide primers for human *Slug* mRNA amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers 5’-3’</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human <em>Slug</em></td>
<td>Forward Primer 1. ATGGCCTCTCTCTCTTTCC</td>
<td>582</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer 2. AGCAGCCAGATTCTCATGT</td>
<td>582</td>
</tr>
<tr>
<td></td>
<td>Mutant Reverse Primer 3. AGCAGCCAGATTCTCATGT GCATCTTCAG</td>
<td>338</td>
</tr>
</tbody>
</table>

582bp target cDNA

Forward Primer ATGGCCTCTCTCTCTCTTTCC AGCAGCCAGATTCTCATGT Reverse Primer

338bp competitor cDNA
Figure 9. Schematic diagram summarizing the QC-PCR strategy employed in the study of Slug. A competitive Slug cDNA (338 bp) was generated through the addition of a stretch nucleotides, corresponding to a specific sequence within the target cDNA (582bp), to the initial reverse primer
FIGURE 10. Schematic diagram of oligonucleotide primers for human *Twist* mRNA amplification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers 5'-3'</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Twist</td>
<td>Forward Primer: AGTCCGCAGTCTTACGAGGA</td>
<td>576</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: GCAGAGGATGAGGATGAGTGT</td>
<td>576</td>
</tr>
<tr>
<td>Mutant Forward Primer: AGTCCGCAGTCTTACGAGGA CAAGTGGCA</td>
<td>361</td>
<td></td>
</tr>
</tbody>
</table>

576bp target cDNA

↑

Forward Primer: AGTCCGCAGTCTTACGAGGA

Reverse Primer: GCAGAGGATGAGGATGAGTGT

361bp competitor cDNA

↓

Mutant Forward Primer: AGTCCGCAGTCTTACGAGGA CAAGTGGCA

Reverse Primer: GCAGAGGATGAGGATGAGTGT
Figure 10. Schematic diagram summarizing the QC-PCR strategy employed in the study of *Twist*. A competitive *Twist* cDNA (361 bp) was generated through the addition of a stretch nucleotides, corresponding to a specific sequence within the target cDNA (576 bp), to the initial forward primer.
sequencing to confirm the specificity of the primers. These clones were also used to generate internal standard *E-cadherin, Snail, Slug* or *Twist* cDNA fragments by standard molecular biology techniques.

### 3.4 Quantitative competitive PCR (QC-PCR)

The QC-PCR strategy employed in these studies is based upon the competitive coamplification of a known amount of a internal standard specific for *E-cadherin, Snail, Slug* or *Twist* that was added to aliquots of the first strand cDNA prepared from the BeWo cell RNA. To determine the optimal amounts of the internal standards to be used in the QC-PCR analyses, PCR mixtures containing 1µl of serial diluted competitive PCR products and 1µl of first strand cDNA. PCR was then performed using these cDNA mixtures and the distinct *E-cadherin, Snail, Slug* or *Twist* primer sets under the optimized conditions described above.

An aliquot (10µl) of the resultant *E-cadherin, Snail, Slug* or *Twist* PCR products containing the resultant target and internal standard PCR products were resolved by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. The intensity of the ethidium bromide staining of the PCR products was analyzed using a UV densitometry imaging system (Biometra, Whiteman Co., Gottingen, Germany). Volume counts (square millimeters) of the scanned PCR products were then determined using Scion Image computer software (Scion Image Co., Frederick, MD). The absorbance values obtained for each of the native and the corresponding competitive cDNAs generated by PCR were plotted against the amount of internal standard initially added to the reaction mixtures. The point of interception on these line graphs was taken as the
optimal amount of internal standard to be used in the QC-PCR analysis. Based upon these observations, E-cadherin, Snail, Slug or Twist internal standard cDNAs were added to aliquots of the first strand cDNA to be used in the QC-PCR analysis at concentrations of \(7.8 \times 10^{-3}\), 0.125, \(3.765 \times 10^{-6}\) and \(1.95 \times 10^{-3}\) pg/\(\mu\)l, respectively. Subsequent QC-PCR reactions were performed using an aliquot (1\(\mu\)l) of the first strand cDNA synthesized from the BeWo cell RNA and containing the optimized amount of the corresponding internal standard cDNA, with the E-cadherin, Snail, Slug or Twist primer sets under the PCR conditions described above. The ratios of the intensity of ethidium bromide staining of the resultant target/internal standard PCR products were determined as described above.

3.5 Western blot analysis

Cultured BeWo cells were washed three times in PBS and incubated at 4°C for 30 min on a rocking platform in 100\(\mu\)l of cell lysis buffer (Biosource International, Carmaillo, CA) containing 10mM Tris, pH 7.4; 100mM NaCl; 1mM EDTA; 1mM EGTA; 1mM NaF; 1% Triton X-100; 10% Glycerol; 0.1% SDS; 0.5% deoxycholate supplemented with 1mM PMSF and protease inhibitor cocktail. The cell lysates were centrifuged at 10,000 x g for 20 min at 4°C, and the supernatants were used for Western blot analysis. The concentration of protein in the cell lysates was determined using the BCA kit (Pierce Chemicals, Rockford, IL). Proteins (40\(\mu\)g) were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane (Hybond-P, Amersham Pharmacia Biotech., Oakville, Canada). Subsequently, membranes were blocked with 5% nonfat dry milk for 1h at room temperature. The nitrocellulose blots were probed with
antibodies directed against human Snail (Abgent, San Diego, CA), Slug (Abgent, San Diego, CA), Twist (Santa Cruz, Biotechnology, Inc.) or E-cadherin (Transduction Laboratory, Lexington, KY) overnight at 4 °C. After washing three times with Tris buffered saline (TBS) containing 0.1% Tween-20, membranes were incubated with 1:2000 secondary antibodies in 5% nonfat dry milk for 1h. The enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech., Oakville, Canada) was used to detect antibody bound to antigen. The blots were then scanned using a LKB laser densitometer.

3.6 Immunolocalization of E-cadherin and Snail in BeWo cell cultures.

Cultures of BeWo cells grown on glass coverslips for 72h were fixed in 4% paraformaldehyde for 30 min at room temperature. The fixed cells were immunostained using a mouse monoclonal antibody directed against E-cadherin (Transduction Laboratory, Lexington, KY)) and a rabbit polyclonal antibody directed against Snail (Abgent, San Diego, CA). Sequential incubations were performed. These included incubations with 10% normal horse serum for monoclonal antibody and normal goat serum for-polyclonal antibody at 37°C for 30 min, primary antibody at 37°C for 1h, secondary biotinylated antibody at 37°C for 30 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three 5 min washes in PBS. The cells were then exposed to chromagen reaction solution (0.0035% diaminobenzidine and 0.03% hydrogen peroxide) for 5 min, washed in tap water for 5 min, counterstained in haematoxylin, dehydrated, cleared, and mounted.
3.7 Immunohistochemical analysis of Snail in vivo

Immunohistochemistry was performed using sections prepared from permanent paraffin blocks containing first trimester chorionic villi (n=3). The tissue sections were immunostained using polyclonal antibodies directed against human Snail (Abgent, San Diego, CA) on three independent occasions. Nonspecific isotype-matched antibodies were used as negative controls. Sequential incubations were performed and included 5% normal goat serum for 30 min, primary antibody for 2 hr at room temperature, three 5 min washes in PBS, secondary antibody conjugated with HRP-labeled polymer (EnVision™; DakoCytomation) for 30 min, and three 5 min washes in PBS. The sections were then developed in NovaRED substrate (Vector Labs, SK-4800) for 8 min, washed in tap water for 5 min, counterstained in hematoxylin, dehydrated, cleared, and mounted.

3.7 Statistical analysis

The absorbance values of the ethidium bromide stained PCR products were subjected to statistical analysis using GraphPad Prism 4 software (San Diego, CA). Statistical differences between the absorbance values were assessed by the 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 (±SEM) obtained from ≥3 different experiments.
PART IV. RESULTS

4.1 Time-dependent effects of 8-bromo-cAMP on E-cadherin mRNA and protein levels in human BeWo choriocarcinoma cells

The Quantitative Competitive Polymerase Chain Reaction (QC-PCR) strategy used in the following studies involves the competitive co-amplification of a known amount of internal standard cDNA specific for the target of interest. To determine the optimal amount of internal standard E-cadherin cDNA to be used in the QC-PCR analysis, serial dilutions of this internal standard cDNA were coamplified in the presence of a fixed amount of target cDNA. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cells with known decreasing amounts of competitive E-cadherin cDNA. As expected, progressively decreasing the amount of the latter competitive cDNA resulted in concomitant increases in the PCR amplification of the BeWo cell target cDNA (Figure. 11). The point of interception of the lines depicting amplified product of internal standard cDNA or target cDNA indicated the optimal amount of internal standard cDNA that should be added to the subsequent QC-PCR reactions. Hence, 7.8x10^{-3} pg/μl of E-cadherin cDNA was added to each QC-PCR reaction (Figure. 11).

The time-dependent effect of a fixed concentration of cAMP on E-cadherin transcript expression was investigated. A concentration of 1.5mM cAMP was chosen for this experiment based on its reported ability to induce BeWo cell differentiation (MacCalman et al., 1996). The E-cadherin mRNA transcript was detected in all of the total RNA extracts prepared from the BeWo cell cultures. E-cadherin transcript level was not affected during culture of the cells for 48h in the absence of 8-bromo-cAMP (Figure.
12A and C). However, QC-PCR revealed a 50% decrease in *E-cadherin* mRNA levels in BeWo choriocarcinoma cells after 12h of culture in the presence of this cAMP concentration (Figure. 12B and C). The levels of this mRNA transcript continued to decrease over time with a 9-fold decrease observed at the termination of these studies at 48h (Figure. 12B and C).
Figure 11. Determination of the optimal amount of internal standard E-cadherin cDNA to be added to the QC-PCR reaction mixtures. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cell cultures and decreasing amounts of competitive E-cadherin cDNA. A representative ethidium bromide-stained gel containing the resultant PCR products is presented (upper panel). The sizes of the resultant target (560bp) and internal standard (305bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. The gels were analyzed by UV densitometry and the volume counts obtained for both PCR products were plotted in the line graph (lower panel). The point of interception of the two lines indicates the optimal amount ($7.8 \times 10^{-3}$pg/μl) of competitive cDNA to be added to each QC-PCR reaction mixture.
Figure 12. Time-dependent inhibition of *E-cadherin* mRNA transcript expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 12, 24, or 48 h. B. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 12, 24, or 48 h. The sizes of the resultant target (560bp) and internal standard (305bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. C. The absorbance ratios of the target QC-PCR product versus that of the internal standard were calculated from three independent studies and were represented in the bar graphs (mean ± SEM, n=3; *p*< 0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
E-cadherin protein expression was examined in BeWo cells treated with 1.5mM cAMP as above. Western blot analysis of protein extracts prepared from the cells was carried out using a mouse monoclonal antibody directed against E-cadherin. This revealed the presence of a single E-cadherin (120 kDa) protein species in all of the cellular extracts (Figure. 13A and B). No significant change in E-cadherin protein expression was detected in cells cultured for up to 72h in the absence of cAMP (Figure. 13A and C). E-cadherin protein levels decreased over time in culture in the presence of cAMP, with a significant 20% reduction observed at 48h (Figure. 13B and C). There was a pronounced decrease of E-cadherin protein levels at 72h of culture as the cells continued to fuse and become multinucleated (Figure. 13B and C). At this time-point, the protein levels of E-cadherin were halved when compared to that of 0h control. The cAMP-induced inhibition of E-cadherin expression was thus observed at both the transcript and protein levels, although protein expression declined more slowly and to a lesser extent than did transcript level. This may reflect differences in the half-life/turnover of E-cadherin protein and mRNA.

The effect of cAMP on E-cadherin expression was also investigated by immunohistochemical staining of BeWo cell cultures. Intense immunostaining for E-cadherin expression was detected in mononucleate BeWo cells cultured in the absence of cAMP (Figure. 14A). In particular, E-cadherin was localized to areas of cell-cell contact in these trophoblastic cells (Figure. 14A, arrows). After 72h culture in the presence of cAMP, E-cadherin immunostaining was not detectable on the surface of the multinucleated syncytium (Figure. 14B, area in dotted square) and was only detected in areas of unfused BeWo cell-cell contacts (Figure. 14B, arrows). Although E-cadherin
immunostaining was still detectable after 72h culture, the overall colour intensity of the E-cadherin staining was significantly reduced compared to that of control cells that were cultured for 72h without 1.5mM 8-bromo-cAMP. Cells immunostained with a nonspecific isotype-matched polyclonal antibody provided a negative control (Figure. 14C).
Figure 13. Time-dependent inhibition of E-cadherin protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Protein lysates were prepared from BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 24, 48, and 72h. Each lysate (10 μg protein) was probed for E-cadherin using anti-E-cadherin monoclonal antibody. B. As in A, except protein lysates were prepared from BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 24, 48, and 72 h. C. The autoradiograms were then scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies for both control and 8-bromo-cAMP treatment were represented (mean ±SEM; n=3) in the bar graphs (*p≤0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
Figure 14. Immunolocalization of E-cadherin in BeWo cell cultures. A. BeWo cells were stained for E-cadherin after 72h culture in the absence of 8-bromo-cAMP. Arrows represent areas of cell-cell contact. B. BeWo cells were stained for E-cadherin after 72h culture in the presence of 1.5mM 8-bromo-cAMP. The dotted square represents multinucleated syncytium and the arrows depict staining of unfused BeWo cells. Immunostaining utilized a monoclonal anti-E-cadherin antibody. C. Negative control in which the primary antiserum was replaced with a non-specific isotype-matched monoclonal antibody.
4.2 Time-dependent effects of 8-bromo-cAMP on Snail mRNA and protein levels in human BeWo choriocarcinoma cells

Cloning and characterization of the human E-cadherin promoter has revealed that two E-boxes present in a proximal fragment of the promoter match the DNA-binding site of Snail (Mauhin et al., 1993; Battle et al., 2000). Snail is known to regulate the expression of E-cadherin in several developmental processes in different species, from fly to human. Drosophila Snail mutant embryos die at the gastrulation stages and show a gastrulation-defective phenotype that has been associated with impaired downregulation of E-cadherin (Oda et al., 1998). In light of these observations, Snail and related proteins could be the fundamental regulators of the E-cadherin-mediated trophoblast differentiation and fusion process. Therefore the expression profiles of Snail and of its other family member Slug were investigated during cAMP-induced differentiation and fusion of human trophoblastic cells.

As described for E-cadherin-specific QC-PCR (Results section 4.1), the optimum amount of internal standard Snail cDNA to be used in the QC-PCR analysis was determined. Serial dilutions of the internal standard cDNA for Snail were coamplified in the presence of a fixed amount of target cDNA. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cells with known decreasing amounts of competitive Snail cDNA (Figure. 15). The point of interception of the lines depicting amplified product of internal standard cDNA or target cDNA indicated the optimal amount of internal standard cDNA that should be added to the subsequent QC-PCR reactions. Hence, 0.125 pg/μl of Snail cDNA was added to each QC-PCR reaction (Figure. 15).
The time-dependent effect of a fixed concentration of cAMP on \textit{Snail} transcript expression was investigated. The \textit{Snail} mRNA transcript was present in all of the total RNA extracts prepared from the BeWo cell cultures. QC-PCR detected a minor increase in \textit{Snail} mRNA level in cells culture over time in the absence of 8-bromo-cAMP (Figure. 16A and C), indicating that \textit{Snail} was slightly upregulated during normal cell growth. QC-PCR detected a significant and continuous increase in \textit{Snail} mRNA level in these trophoblastic cells with increasing time in culture in the presence of 1.5mM cAMP (Figure. 16B and C). There was about a 6-fold increase of this mRNA transcript at 24h of culture in the presence of 8-bromo-cAMP when compared to the 0h control (Figure. 16C). The levels of \textit{Snail} mRNA transcript continued to increase with time in culture and an approximate 9-fold increase in transcript level (compared to 0h of culture) was apparent after 48h of culture in the presence of 1.5mM 8-bromo cAMP (Figure. 16C).
Figure 15. Determination of the optimal amount of internal standard Snail cDNAs to be added to the QC-PCR reaction mixtures. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cell cultures and decreasing amounts of competitive Snail cDNA. A representative ethidium bromide-stained gel containing the resultant PCR products is presented (upper panel). The sizes of the resultant target (552bp) and internal standard (351bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. The gels were analyzed by UV densitometry and the volume counts obtained for both PCR products were plotted in the line graph (lower panel). The point of interception of the two lines indicates the optimal amount (0.125 pg/μl) of competitive cDNA to be added to each QC-PCR reaction mixture.
Figure 16. Time-dependent induction of Snail mRNA transcript expression in BeWo choriocarcinoma cells by 8-bromo-cAMP. A. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 12, 24, or 48 h. B. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 12, 24, or 48 h. The sizes of the resultant target (552bp) and internal standard (351bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. C. The absorbance ratios of the resultant target QC-PCR product versus that of the internal standard were calculated from three independent studies and were represented in the bar graphs (mean ± SEM, n=3; *p< 0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
A

M    0    12    24    48

552bp
351bp

B

M    0    12    24    48

552bp
351bp

C

Time in culture (h)

Target/internal standard

■ control
□ treated with 8-bromo-cAMP

0  0.5  1  1.5  2  2.5  3  3.5  4  4.5

0  12  24  48
Snail protein expression was examined in BeWo cells treated with 1.5mM cAMP as above. Western blot analysis of protein extracts prepared from the cells was carried out using a rabbit polyclonal antibody directed against human Snail. This revealed the presence of a major Snail (29 kDa) protein species after 24h and 48h of culture (Figure 17B). There was no detectable Snail protein expression in the 0h and 12h control cultures (Figure 17A) or in 0h and 12h 8-bromo-cAMP-treated cells (Figure 17B). In accord with the increase in Snail mRNA level that was detected in cells cultured for 24h and 48h in the absence of cAMP (Figure 17A and C), the protein expression of Snail was also slightly increased upon 24h and 48h of culture without 8-bromo-cAMP (Figure 17A). When cultured in the presence of cAMP, Snail protein expression was not observed until 24h (Figure 17B). In agreement with the QC-PCR data, the late expression levels of the Snail protein were significantly increased, with an 8-fold increase observed at 48h culture in response to 8-bromo-cAMP treatment (Figure 17C).

The effect of cAMP on Snail expression was also investigated by immunohistochemical staining of BeWo cell cultures. Snail immunostaining was barely detectable in BeWo cells cultured in the absence of 1.5mM 8-bromo-cAMP (Figure 18A). Intense Snail immunostaining was detected in BeWo cells after treatment with 1.5mM 8-bromo-cAMP. In particular, Snail was localized to the nucleus in these trophoblastic cells after 72h culture in the presence of cAMP (Figure 18B, arrows). Cells immunostained with a nonspecific isotype-matched polyclonal antibody provided a negative control (Figure 18C).

The immunolocalization of Snail was also examined in the villous and syncytial trophoblast layers of the first-trimester placenta. Snail expression was mainly
immunolocalized to syncytial trophoblasts of first-trimester chorionic villi (Figure 19A). In particular, the expression of Snail appeared to be localized primarily to the nucleus of the syncytial trophoblasts (Figure 19A, arrows). Snail was not found in the mesenchymal core or blood vessels of these chorionic villi. Sections immunostained with a nonspecific isotype-matched polyclonal antibody were used as a negative control (Figure 19B).
Figure 17. Time-dependent induction of Snail protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Protein lysates were prepared from BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 24, 48, and 72 h. Each lysate (40 μg protein) was probed for Snail using anti-Snail polyclonal antibody. B. As in A, except protein lysates were prepared from BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 24, 48, and 72 h. C. The autoradiograms were then scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies of control and 8-bromo-cAMP treatment were represented (mean ±SEM; n=3) in the bar graphs (*p<0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
0  24  48  72

29kDa

B

0  24  48  72

29kDa

C

Relative protein expression (arbitrary units)

0  0.5  1  1.5  2  2.5  3

Time in culture (h)

■ control

□ treated with 8-bromo-cAMP

71
Figure 18. Immunolocalization of Snail in BeWo cell cultures. A. BeWo cells were stained for Snail after 72h culture in the absence of 8-bromo-cAMP. B. BeWo cells were stained for Snail after 72h culture in the presence of 1.5mM 8-bromo-cAMP. Immunostaining utilized a polyclonal anti-Snail antibody. Arrows indicate nuclear staining. C. Negative control in which the primary antiserum was replaced with a non-specific isotype-matched monoclonal antibody.
Figure 19. Immunolocalization of Snail in the villous and syncytiotrophoblast layers of the first-trimester placenta. A. Paraffin sections of first-trimester (gestational ages 8-12 week, n=3) chorionic villi were immunostained with polyclonal antibodies directed against Snail. Arrows represent syncytiotrophoblasts (ST) and cytotrophoblasts (CT). B. Sections immunostained with a non-specific isotype-matched polyclonal antibody were used as negative control. Arrows represent syncytiotrophoblasts (ST) and cytotrophoblasts (CT). Immunohistochemical analysis of these placental tissues was performed on three independent occasions.
4.3 Time-dependent effects of 8-bromo-cAMP on Slug mRNA and protein levels

The cAMP-regulated expression profile of Slug, a Snail family member, was also investigated in BeWo cells. As described above for E-cadherin QC-PCR (Results section 4.1) and Snail QC-PCR (Results section 4.2), the optimum amount of internal standard Slug cDNA to be used in QC-PCR analysis was determined. Serial dilutions of the internal standard cDNA for Slug were coamplified in the presence of a fixed amount of target cDNA. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cells with known decreasing amounts of competitive Slug cDNA. However, progressively decreasing the amount of the latter competitive cDNA did not result in concomitant increases in the PCR amplification of the BeWo cell target cDNA (Figure. 20). This indicated that the Slug mRNA transcript was expressed at a very low level (10^{-6} pg/μl), and that even decreasing the amount of the competitive cDNA to low levels was unable to promote increased Slug cDNA amplification. Though there was no clear equivalence between the target QC-PCR product and that of the internal standard, 3.765x10^{-6} pg/μl of Slug cDNA was chosen as the amount of internal standard cDNA to use in subsequent QC-PCR experiments.

As expected the levels of the mRNA transcript encoding Slug remained relatively low in the absence of 8-bromo-cAMP (Figure. 21A and C). Similarly, the mRNA levels of Slug remained fairly constant and low even in the presence of 1.5mM 8-bromo-cAMP, at least at the time points examined in these studies (Figure. 21B and C).

Slug protein expression was examined in BeWo cells treated with 1.5mM cAMP as above. Western blot analysis of protein extracts prepared from the cells was carried out using a rabbit polyclonal antibody directed against Slug. This revealed the presence of an
immunoreactive band of 29 kDa in all of the cellular extracts (Figure. 22A and B). In accord with the QC-PCR analysis, the protein levels of Slug remained fairly constant and low in both control and in the presence of 8-bromo-cAMP (Figure. 22C).
Figure 20. Determination of the optimal amount of internal standard Slug cDNAs to be added to the QC-PCR reaction mixtures. QC-PCR was performed under optimized conditions using an aliquot (1 µl) of first strand cDNA synthesized from RNA of BeWo cell cultures and decreasing amounts of competitive Slug cDNA. A representative ethidium bromide-stained gel containing the resultant PCR products is presented (upper panel). The sizes of the resultant target (582 bp) and internal standard (338 bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. The gels were analyzed by UV densitometry and the volume counts obtained for both PCR products were plotted in the line graph (lower panel). The point of interception of the two lines indicates the optimal amount \(3.765 \times 10^{-6} \text{pg/µl}\) of competitive cDNA to be added to each QC-PCR reaction mixture.
Figure 21. Time-dependent effect of Slug mRNA transcript expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 12, 24, or 48 h. B. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 12, 24, or 48 h. The sizes of the resultant target (582bp) and internal standard (338bp) PCR products relative to a 100-bp ladder (lane M) are marked to the right of the photomicrographs. C. The absorbance ratios of the resultant target QC-PCR product versus that of the internal standard were calculated from three independent studies and were represented in the bar graphs (mean ± SEM, n=3).
Figure 22. Time-dependent effect of Slug protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Protein lysates were prepared from BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 24, 48, and 72h. Each lysate (40μg protein) was probed for Slug using anti-Slug polyclonal antibody. B. As in A, except protein lysates were prepared from BeWo cell cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 24, 48, and 72h. C. The autoradiograms were then scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies of control and 8-bromo-cAMP treatment were represented (mean±SEM; n=3) in the bar graphs (*p≤0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
4.4 Time-dependent effects of 8-bromo-cAMP on Twist mRNA and protein levels

In both tumor metastasis and trophoblast differentiation, there is a disappearance of E-cadherin at the cell-cell junction. This allows the former cells to dissociate from one another and become invasive, and for the later cells to fuse. Recently, the transcription factor *Twist*, which has long been implicated in embryonic morphogenesis, was found to be a master regulator in tumor metastasis. Ectopic expression of *Twist* results in loss of E-cadherin-mediated cell-cell adhesion and induction of cell motility (Yang *et al.*, 2004). It is speculated that Twist may be regulated during cadherin-mediated differentiation of the human trophoblastic cells. Therefore the expression profile of *Twist* was investigated during cAMP-induced differentiation and fusion of BeWo cells.

As described above for *E-cadherin* QC-PCR (Results section 4.1) and *Snail* QC-PCR (Results section 4.2), the optimum amount of internal standard *Twist* cDNA to be used in QC-PCR analysis was determined. Serial dilutions of the internal standard cDNA for *Twist* were coamplified in the presence of a fixed amount of target cDNA. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cell cultures with known decreasing amounts of competitive *Twist* cDNA. As expected, progressively decreasing the amount of the latter competitive cDNA resulted in concomitant increases in the PCR amplification of the BeWo cell target cDNA (Figure 23). The point of interception of the lines depicting amplified product of internal standard cDNA or target cDNA indicated the optimal amount of internal standard cDNA that should be added to the subsequent QC-PCR reactions. Hence, 1.95x10^-3 pg/μl of *Twist* cDNA was added to the subsequent QC-PCR reactions (Figure 23).
The time-dependent effect of a fixed concentration of cAMP on Twist transcript expression was investigated. QC-PCR revealed about a 2-fold increase in Twist mRNA levels in BeWo cell after 24h of culture in the presence of 1.5mM 8-bromo-cAMP (Figure 24B and C). The levels of this mRNA continued to increase over time as the BeWo cells underwent terminal differentiation and fusion, until the termination of these studies at 48h (Figure 24B and C). On the other hand, the mRNA level of Twist was slightly upregulated in the absence of 8-bromo-cAMP as BeWo cells continued to grow in culture (Figure 24A).
Figure 23. Determination of the optimal amount of internal standard Twist cDNAs to be added to the QC-PCR reaction mixtures. QC-PCR was performed under optimized conditions using an aliquot (1μl) of first strand cDNA synthesized from RNA of BeWo cell cultures and decreasing amounts of competitive Twist cDNA. A representative ethidium bromide-stained gel containing the resultant PCR products is presented (upper panel). The sizes of the resultant target (576bp) and internal standard PCR (361bp) products relative to 100-bp ladder (lane M) are marked to the right of the photomicrographs. The gels were analyzed by UV densitometry and the volume counts obtained for both PCR products were plotted in the line graph (lower panel). The point of interception of the two lines indicates the optimal amount of (1.95x10^{-3}pg/μl) competitive cDNA to be added to each QC-PCR reaction mixture.
Figure 24. Time-dependent induction of Twist mRNA transcript expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA prepared from BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 12, 24, or 48 h. B. Representative photomicrograph of an ethidium bromide-stained gel containing QC-PCR products generated using template cDNA synthesized from RNA of BeWo cells cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 12, 24, or 48 h. The sizes of the resultant target (576bp) and internal standard (361bp) PCR products relative to a 100-bp ladder (lane M) were marked to the right of the photomicrographs. C. The absorbance ratios of the resultant target QC-PCR product versus that of the internal standard were calculated from three independent studies and were represented in the bar graphs (mean ± SEM, n=3; *p< 0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
Twist protein expression was examined in BeWo cells treated with 1.5mM cAMP as above. Western blot analysis of protein extracts prepared from the cells was carried out using a rabbit polyclonal antibody directed against Twist. This revealed the presence of an immunoreactive band of 32 kDa (the expected size of Twist) in all of the cellular extracts (Figure 25A and B). Western blot analysis revealed that 1.5mM 8-bromo-cAMP induced a 3-fold increase in the protein expression of Twist at 72h (Figure 25C). In accord with the QC-PCR analysis, an increase in Twist protein expression was detected in the absence of 8-bromo-cAMP as the cells continued to grow but to a much lesser extent than that observed in the presence of 1.5mM 8-bromo-cAMP (Figure 25C).
Figure 25. Time-dependent induction of Twist protein expression in BeWo choriocarcinoma cells by 1.5mM 8-bromo-cAMP. A. Protein lysates were prepared from BeWo cells cultured in the absence of 8-bromo-cAMP for 0, 24, 48, and 72h. Each lysate (40μg protein) was probed for Twist using anti-Twist polyclonal antibody. B. As in A, except protein lysates were prepared from BeWo cell cultured in the presence of 1.5mM 8-bromo-cAMP for 0, 24, 48, and 72h. C. The autoradiograms were then scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies of control and 8-bromo-cAMP treatment were represented (mean±SEM; n=3) in the bar graphs (*p≤0.05 vs. 0h control). Asterisks depict significant differences with the 0h control.
PART V. DISCUSSION

Syncytial fusion is a very rare event in cell biology and obligatory to embryonic development. In humans, there are 4 typical cell-cell fusion events that have been well studied: the fertilization of the egg by sperm (Wilson and Snell, 1998), the formation of myotubes from mononucleate myoblasts during striated muscle development (Nadal-Grinard, 1978), the differentiation of osteoclasts (Roodman, 1996;) and the formation of the syncytial trophoblast layer during human placentation (Kilman et al., 1986). All of these syncytia are highly specialized endpoints of particular differentiation pathways. Membrane fusion processes play an important role in the vesicular transport system that mediates the synthesis and trafficking of proteins (Rothman, 1996; Rothman and Wieland, 1996) and one of the prerequisites of these fusion events is to establish extensive adhesive interactions. Obviously, cell-cell fusion not only changes the cell morphology but also the physiology of these cells. For example, from an endocrine point of view, syncytial fusion of cytotrophoblasts is not just a morphological event but is associated with increased hormone secretion (Hoshina et al., 1982; Cronier et al., 1994).

5.1 Cadherin subtype switching during trophoblast terminal differentiation

It is well accepted that morphogenesis and cell differentiation depend, in part, on the regulated expression of adhesion molecules. Some of these adhesion molecules have been well characterized including E-cadherin (Coutifaris et al., 1991) and Cadherin-11 (MacCalman, 1997). Previous studies have demonstrated that the expression levels of E-cadherin, in conjunction with those of its associated cytoplasmic proteins, α-, β-, γ-catenin, and p120^CTN, are down-regulated during the terminal differentiation of
trophoblasts \textit{in vitro} and \textit{in vivo} (Getsios \textit{et al.}, 2003). In contrast, an increase in Cadherin-11 expression is capable of promoting the formation of multinucleated syncytia in mononucleate trophoblastic cell cultures.

\section*{5.2 E-cadherin expressions during cAMP-induced differentiation of BeWo cells}

The results of the present study that were obtained using QC-PCR revealed a significant decline in \textit{E-cadherin} mRNA levels in BeWo choriocarcinoma cells after 12h of culture in the presence of 1.5mM 8-bromo-cAMP. The levels of this mRNA transcript continued to decline until the termination of these studies at 48h. Consistent with the transcript reduction, there was a decrease in \textit{E-cadherin} protein expression as the BeWo cells continued to undergo differentiation and fusion in response to 8-bromo-cAMP treatment. No significant changes in \textit{E-cadherin} transcript and protein levels were detected in cells cultured for up to 72h in the absence of cAMP. Although there is strong evidence that the process of trophoblast terminal differentiation involves the down-regulation of \textit{E-cadherin}, the mechanisms underlying this \textit{E-cadherin} repression are still poorly understood. The regulation of \textit{E-cadherin} expression occurs mainly at transcriptional levels. Recent cloning and characterization of the human \textit{E-cadherin} promoter has revealed that two E-boxes present in a proximal fragment of the promoter are important for the repression of this gene (Giroldi \textit{et al.}, 1997). The two E-boxes characterized so far in the human and mouse \textit{E-cadherin} promoters have an identical core, consisting of the sequence 5'CACCTG. This sequence exactly matches the DNA-binding site of \textit{Snail} (Mauhin \textit{et al.}, 1993; Battle \textit{et al.}, 2000).
5.3 Potential role of the Snail/Slug family during cAMP-induced differentiation of BeWo cells

The Snail/Slug family was first identified in Drosophila, where it downregulates the transcription of E-cadherin to control gastrulation. Recently, direct evidence showed that the Snail transcription factor and its family protein Slug repress E-cadherin in human epithelial tumor cell lines, including hepatocellular carcinoma (Sugumachi et al., 2003) and breast carcinoma (Hajra et al., 2002). Indeed, loss of E-cadherin expression has been shown to be responsible for the loss of intercellular adhesion occurring during invasion and metastasis (Battle et al., 2000).

The results of the present study demonstrated that Snail mRNA level and protein expression were low in the BeWo choriocarcinoma cells cultured in the absence of 8-bromo-cAMP, and that Snail mRNA level and protein expression were significantly increased after adding 8-bromo-cAMP to the culture medium. Both the mRNA and protein levels continued to increase as the cells were induced to differentiate. The induction of Snail protein was observed to be slower than that of Snail transcript, however, the late expression levels of the Snail protein at 72h matched the maximal decline of E-cadherin protein levels. The coordinated expression of Snail and E-cadherin may thus be inter-related. As 8-bromo-cAMP was able to induce fusion and differentiation of the BeWo cells in conjunction with the down-regulation of E-cadherin, the results of our study suggest that the disappearance of E-cadherin from the cAMP-induced trophoblastic cell fusion was through up-regulation of Snail gene and protein expression. To our knowledge, this is the first time that this inverse relationship between Snail and E-cadherin mRNA and protein levels has been demonstrated during cAMP-
induced trophoblast cell fusion. This observation indicates that *Snail* is a likely negative regulator of E-cadherin expression during trophoblast cell differentiation. Moreover, immunostaining studies revealed that *Snail* was localized in the nucleus after the cells were induced to fuse and differentiate. According to Dominguez *et al.* (2003) the activity of Snail is regulated by the phosphorylation and subcellular location. Under normal conditions *Snail* is phosphorylated and stays in the cytoplasm and is not able to access its target promoters. Therefore, *Snail* promotes its effects only when it is upregulated to levels where it cannot be totally phosphorylated and hence some *Snail* is retained in the nucleus and subsequently binds to the target gene. Our results demonstrate intense immunostaining of *Snail* in the nucleus of the cAMP-treated cells, thus it is highly likely that this *Snail* protein is in its functional state and able to bind to its target sequences, including those of the *E-cadherin* gene.

Recently, Zhou *et al.* (2004) found that *Snail* contains two glycogen synthase kinase 3β (GSK3β) phosphorylation motifs. Immunocomplex kinase assays showed that GSK3β physically interacts with *Snail* and specifically phosphorylates it at two consensus motifs. This finding further confirms the role of phosphorylation in the regulation of *Snail* activity. Under normal conditions, GSK3β phosphorylates *Snail* in the nucleus and this directs *Snail* to the cytoplasm. Inhibition of the kinase activity of GSK3β by phosphorylation stabilizes *Snail*, and unphosphorylated *Snail* is retained in the nucleus. This results in the upregulation of *Snail* protein in the nucleus. What are the upstream pathways that regulate the activity of GSK3β, and in turn control the phosphorylation of *Snail*, thus regulating its subcellular localization? According to Li *et al.* (2000), elevation of intracellular cAMP levels in rat cerebellar granule neurons leads
to phosphorylation and inhibition of GSK3β. The increased phosphorylation of GSK3β by protein kinase A (PKA) occurs at serine 9 and purified PKA is able to phosphorylate recombinant GSK3β in vitro. These findings agree with our working model that cAMP, which we used to induce BeWo cell differentiation, activates PKA and PKA in turn phosphorylates GSK3β to effect the upregulation of Snail protein in the nucleus (Figure 26).

In contrast to Snail, the mRNA level and protein expression of Slug, the other member of the Snail protein family, remained relatively low and constant when BeWo cells were cultured in the absence or presence of 8-bromo-cAMP. Although Snail and Slug are members of the same protein superfamily and share a high degree of homology, there are functional differences between them. For example, Slug has been implicated in triggering EMT in the chick by repressing E-cadherin expression (Nieto et al., 1994), however, a role for Slug of repressor for E-cadherin expression has not been supported in other models. The functional differences between Snail and Slug in respect to E-cadherin regulation likely reflect their specific contributions in different cell types and different species.

5.4 Potential role of Twist in repressing E-cadherin expression

In both tumor metastasis and trophoblast differentiation, there is a disappearance of E-cadherin from the cell-cell junctions. This allows the former cells to dissociate from one another and become invasive, and the latter cells to fuse. Recently, the transcription factor Twist, which has long been implicated in embryonic morphogenesis, was found to
Figure 26. Proposed signaling pathways that regulate the subcellular localization of Snail in BeWo cells. Under normal conditions, Snail is phosphorylated by GSK3β and transported to the cytoplasm. However, an increase in intracellular cAMP activates PKA which phosphorylates and inhibits GSK3β kinase activity, and this in turn stabilizes Snail to retain Snail in the nucleus.
be a key regulator in tumor metastasis (Yang et al., 2004). Ectopic expression of Twist results in loss of E-cadherin-mediated cell-cell adhesion and induction of cell motility. In human breast cancers, a high level of Twist expression correlates with invasive lobular carcinoma, a highly invasive tumor type associated with loss of E-cadherin expression (Thiery and Morgan, 2004).

Our present studies demonstrate that the mRNA and protein levels of Twist were both significantly upregulated when BeWo cells were induced to fuse and differentiate in response to 8-bromo-cAMP treatment. Unlike the expression pattern of Snail in which the Snail protein was only detected in cAMP-treated cells, Twist protein was found in cells cultured in either the absence or presence of cAMP. As Twist was also slightly upregulated during normal cell culture indicating that the upregulation of Twist was not only a result of cAMP treatment. However, cAMP treatment did reinforce the induction of Twist protein during BeWo cells undergoing fusion and differentiation. As this is the first investigation of the expression profile of Twist during trophoblast cell fusion and differentiation, additional studies are required to understand the underlying mechanism(s) involved in Twist upregulation. It is also of interest to identify the transcriptional targets of Twist. A well-known target gene of Twist is E-cadherin. Interestingly, cAMP-induced increase in Twist protein expression matched the maximal decline of E-cadherin protein levels. This observation is consistent with the hypothesis that Twist is one of the modulators of the expression of E-cadherin during trophoblast fusion and differentiation.
5.5 Future studies

Our findings suggest that Snail and Twist play critical roles in the fusion and differentiation of trophoblastic cells. These observations not only characterize molecular mechanism(s) underlying the reduced E-cadherin expression observed during trophoblast fusion and differentiation, but also provide a basis for further studies to examine the aberrant development of the placenta during pregnancy. As Snail and Twist are found to participate in trophoblast fusion and differentiation, other molecular components of their associated signaling pathways are good candidates to be examined for their possible involvement in trophoblast terminal differentiation. Since the majority of the biological functions of the human placenta are performed by the syncytial trophoblast layer, impaired trophoblastic cell fusion has been correlated with several complications of pregnancy, for example, preeclampsia and Down’s syndrome. Therefore, it is of interest to examine whether aberrant expression of these two transcription factors, Snail and Twist, contributes to the development of persistent trophoblastic disease in vivo. In the future, a siRNA approach will be utilized to examine the effects of abrogated Snail and/or Twist expression on the morphological expression of E-cadherin and on the trophoblast differentiation process. As the mechanisms that regulate Snail remain elusive, future studies must aim to elucidate the signaling pathways underlying the expression and activity of Snail during cAMP-induced differentiation of BeWo choriocarcinoma cells by examining the expression profile of GSK3β and other possible signaling pathways, for example, Wnt signaling.
PART VI. SUMMARY

These studies are the first to demonstrate an increase in the expression of *Snail* and *Twist* during the fusion and differentiation of human trophoblast cells. The altered expression patterns of these two transcription factors correlate with the reduced E-cadherin expression during cAMP-induced fusion and differentiation of human choriocarcinoma BeWo cells. As 8-bromo-cAMP is able to induce fusion and differentiation of the BeWo cells mediated by down-regulation of E-cadherin, the results of our study suggest that the disappearance of E-cadherin from the cAMP-induced trophoblastic cells during fusion is mediated by up-regulation of *Snail* and *Twist*. To our knowledge, this is the first time that this inverse relationship between *Snail*, *Twist* and E-cadherin mRNA and protein levels has been demonstrated during cAMP-induced trophoblast cell fusion. Collectively, these studies not only further our understanding of the cellular mechanisms that mediate the formation and organization of the human placenta but also provide useful insight into the potential roles of these embryonic transcription factors in the trophoblast differentiation process.
PART VII. REFERENCES


regulates the subcellular location and activity of the snail transcriptional repressor.


• Ignotz RA, Massague J (1985) Type β transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. Proc Natl Acad Sci USA 82:8260-8210.


• Panigel M, Myers RE (1972) Histological and ultrastructural changes in rhesus monkey placenta following interruption of fetal placenta circulation by fetectomy or interplacental umbilical vessel ligation. Acta Anat 81:481-506.


• Rubin CS (1994) A kinase anchor proteins and the intracellular targeting of signals carried by cyclic AMP. Biochim Biophys Acta 1224:467-479.
• Skinner MK, Keski-Oja, Osteen KG, Moses HL (1987) Ovarian thecal cells produce transforming growth factor β which can regulate granulosa cell growth. Endocrinology 121:786-792.


