# CADHERIN-MEDIATED DIFFERENTIATION AND FUSION OF HUMAN TROPHOBLASTIC CELLS IN VITRO

by

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# ABSTRACT

The placenta supports fetal growth and development during pregnancy. A key step in human placentation involves the terminal differentiation and fusion of villous cytotrophoblasts to form the multinucleated syncytial trophoblast. To date, the cellular mechanism(s) that mediate this developmental process remain poorly characterized. We have determined that the expression of the calcium-dependent cell adhesion molecule, known as cadherin-11, increases during the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta, BeWo choriocarcinoma cells cultured in the presence of cyclic AMP, and primary cultures of human extravillous cytotrophoblasts treated with transforming growth factor-β1. These observations led us to hypothesize that cadherin-11 mediates the formation of multinucleated syncytium from mononucleate trophoblastic cells *in vitro*.

As cadherin function is regulated by interactions with the cytoplasmic proteins, known as the catenins, we first examined α-, β-, γ-catenin, and $p120^{ctn}$ expression in primary cultures of human villous cytotrophoblasts. The terminal differentiation and fusion of these trophoblastic cells was associated with a reduction in the expression of these four catenin subtypes. In contrast, α-, β-, γ-catenin, and $p120^{ctn}$ were maintained in non-fusing JEG-3 choriocarcinoma cells. These four catenin subtypes were subsequently immunolocalized to the mononucleate cells but not the multinucleated syncytium present in these trophoblastic cell cultures and the villous cytotrophoblasts of the human placenta.

To better define the role(s) of cadherin-11 in the terminal differentiation and fusion of human trophoblastic cells *in vitro*, we examined the effects of ectopic cadherin-11 expression on the morphological differentiation of non-fusing JEG-3 cells. Cadherin-11 expression in mononucleate JEG-3 cells promoted the terminal differentiation and fusion of these cells with time in culture. In contrast, a reduction in cadherin-11 expression was capable of inhibiting the formation of multinucleated syncytium in primary cultures of human villous cytotrophoblasts.

Collectively, these studies demonstrate a critical role for cadherin-11 in the terminal differentiation and fusion of human mononucleate trophoblastic cells *in vitro*. These observations further our understanding of the adhesive mechanisms operative during the formation and organization of the human placenta and provide insight into the cell biology of cadherin-11.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| APC | Adenomatous polyposis coli |
| *arm* | Armadillo |
| ARVCF | *arm* repeat protein deleted in velo cardio facial syndrome |
| BCA | Bicinchonic acid |
| BSA | Bovine serum albumin |
| BrdU | 5-bromo-2'-deoxy-uridine |
| $^{o}C$ | Degrees centigrade |
| $Ca^{2+}$ | Calcium |
| $CaCl_2$ | Calcium chloride |
| Cad | Cadherin |
| CAM | Cell adhesion molecule |
| cAMP | Cyclic adenosine 3',5'-monophosphate |
| CAR | Cell adhesion recognition |
| cDNA | Complementary DNA |
| CEA | Carcinoembryonic antigen |
| CHO | Chinese hamster ovary |
| cm | Centimeter |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| CNR | Cadherin-related neuronal receptor |
| CP | Cytoplasmic |
| CSF-1 | Colony stimulating factor-1 |
| Cx | Connexin |
| DMEM | Dulbecco's Modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| Dsc | Desmocollin |
| Dsg | Desmoglein |
| E2 | 17β-estradiol |
| EC | Extracellular |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamine tetra-acetate |
| EGF | Epidermal growth factor |
| FCS | Fetal calf serum |
| Fn | Fibronectin |
| g | Grams |
| *g* | Gravity |
| GPI | glycosyl phosphatidylinositol |
| h | Hours |
| $H_2O_2$ | Hydrogen peroxide |
| hCG | Human chorionic gonadotropin |
| $HCO_3^-$ | bicarbonate |
| HEPES | 4-(2-hydroxethyl)-1-piperazinesulfonic acid |

| | |
|---|---|
| HERV | Human endogenous retrovirus |
| HUVEC | Human umbilical endothelial cells |
| Ig | Immunoglobin |
| IGF | Insulin-like growth factor |
| IGFBP-1 | IGF binding protein-1 |
| kb | Kilobases |
| kDa | Kilodaltons |
| Ki67 | Nuclear cell proliferation-associated epitope |
| *lacZ* | β-galactosidase |
| LAR-PTP | Leukocyte related-PTP |
| LEF | Lymphocyte Enhancing factor |
| Ln | Laminin |
| MDCK | Madine Darby canine kidney |
| $Mg^{2+}$ | Magnesium |
| min | Minutes |
| ml | Milliliter |
| mM | Millimolar |
| MMP | Matrix metalloproteinase |
| MT-MMP | Membrane-type MMP |
| $M_r$ | Molecular weight |
| mRNA | Messenger RNA |
| µl | Microliter |
| µM | Micromolar |
| $Na^+$,$K^+$-ATPase | Sodium, potassium-adenosine triphosphatase |
| nM | Nanomolar |
| NaCl | Sodium chloride |
| NP-40 | Nonidet P-40 |
| OCT | Optimal Cutting Temperature |
| *P* | Probability |
| P4 | Progesterone |
| PA | Plasminogen activator |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI | PA inhibitor |
| PBS | Phosphate-buffered saline |
| Pcdh | Protocadherin |
| PCNA | Proliferating cell nuclear antigen |
| PDGF | Platelet-derived growth factor |
| PMSF | Phenylmethyl sulfonyl fluoride |
| PTP | Protein tyrosine phosphatase |
| PTP1B-LP | PTP1B-like phosphatase |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal RNA |
| RT | Room temperature |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| S | Svedberg unit of flotation |
| SDS | Sodium dodecyl sulfate |

| | |
|---|---|
| SEM | Standard error of the mean |
| *src* | Rous sarcoma virus |
| SSPE | Standard saline phosphate EDTA |
| SV40 | Simian virus 40 |
| TCF | Thymocyte cell factor |
| TGF-β | Transforming growth factor-β |
| TIMP | Tissue inhibitor of MMP |
| TM | Transmembrane |
| tPA | tissue-type PA |
| Tris-HCL | Tris (hydroxymethyl)-aminomethane-Hydrochloric acid |
| uPA | Urokinase-type PA |
| VEGF | Vascular endothelial growth factor |
| WT1 | Wilms' tumour suppressor protein-1 |
| X-gal | 5-bromo-4-chloro-3-ondolyl-β-D-galactosidase |
| ZO | Zonula occludens |

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# CHAPTER I: OVERVIEW

## 1.1: Introduction

The human placenta plays a key role in regulating the growth, development, and survival of the fetus during pregnancy (Boyd and Hamilton, 1970; Aplin, 1991). Abnormal placental development is thought to be a major underlying cause of several developmental disorders including spontaneous abortion, intrauterine growth retardation, and preeclampsia (Benirschke and Kaufmann, 2000). For example, errors in the formation of this transient organ are associated with fetal demise within the first two months of gestation as is observed in pregnancies terminating in spontaneous abortion (Salafia *et al.*, 1993; van Lijnschoten *et al.*, 1994). Similarly, aberrant placental structure and function are observed in cases of intrauterine growth retardation and preeclampsia, both of which have deleterious effects on the maturation of the fetus (Krebs *et al.*, 1996; Macara *et al.*, 1996). The physiological role of the placenta may extend well beyond the gestational period, as recent studies have correlated the size of this organ at birth with the onset and incidence of specific diseases in adults (Barker *et al.*, 1990; Barker, 1995).

Placental development and function is dependent on the proliferation, differentiation, and invasion of embryonic trophoblastic cells in the maternal endometrium (Aplin, 1991). These specialized cells not only physically anchor the placenta to the uterine wall but also establish a physiologically active interface between the mother and fetus throughout pregnancy. To date, most studies have focused on the ability of gene mutations in mice to adversely effect placental development and trophoblast differentiation in these animals. These studies have indirectly implicated the products of several genes in the developmental processes that lead to embryonic mortality and intrauterine growth retardation in the human (Cross *et al.*, 1994; Cross, 2000). Despite significant advances in our understanding of murine placental development, the cellular

mechanism(s) that regulate trophoblast differentiation during the formation and organization of the human placenta remain poorly characterized.

The calcium ($Ca^{2+}$)-dependent cell adhesion molecule (CAM), cadherin (cad)-11, has been previously detected in the human placenta and correlated with the terminal differentiation and fusion of human trophoblastic cells *in vitro* and *in vivo* (MacCalman *et al.*, 1996a). The main objectives of these studies were to gain insight into the role(s) of cad-11 in this developmental process. As a first step in addressing these outstanding issues, we examined the expression of the cadherin-associated proteins, known as the catenins, during the terminal differentiation and fusion of human trophoblastic cells. These cytoplasmic proteins are believed to be key regulators of cadherin-mediated interactions (Aberle *et al.*, 1996; Gumbiner, 2000). The ability of cad-11 to regulate the terminal differentiation and fusion of cultured trophoblastic cells was subsequently examined.

In this section, the development of the human placenta will be described, with particular emphasis on trophoblast differentiation *in vivo* and *in vitro*. The cellular mechanisms that are believed to modulate trophoblast differentiation and invasion will then be discussed. Finally, the cell biology of the cadherin gene superfamily will be reviewed along with the expression patterns of the different cadherin subtypes during the terminal differentiation and fusion of human trophoblastic cells.

## 1.2: Human implantation and placentation

### *1.2.1: Placental development and trophoblast differentiation in vivo*

The first cell lineage that can be distinguished in the pre-implantation blastocyst is an outer trophectodermal cell layer that gives rise to the epithelial cells of the human placenta

(Hertig *et al.*, 1956; Boyd and Hamilton, 1970). During implantation, the trophectodermal cells undergo apposition, attachment, and penetration through the surface epithelial cells and basement membrane of the maternal endometrium, allowing for the burrowing of the blastocyst into the uterine wall (Schlafke and Enders, 1975; Bentin-Ley *et al.*, 2000). These stages of development are critical to the establishment of a successful pregnancy. It is estimated that between 30% and 70% of human embryos are lost at this time of implantation (Cooke, 1988; Wilcox *et al.*, 1988).

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

The syncytial trophoblast is a terminally differentiated cell formed by the post-mitotic fusion of the underlying villous cytotrophoblasts (Richart, 1961; Kliman *et al.*, 1986). The cellular basis for this terminal differentiation process was first suggested in 1887 by Langhans in

his morphological descriptions of the villous cytotrophoblasts present in the human term placenta (Boyd and Hamilton, 1970). Alternatively, it has been proposed that the development of the multinucleated syncytial trophoblast occurs as a result of nuclear duplication in the absence of cytokenesis (referred to as endomitosis) instead of a cellular fusion process (Sarto *et al.*, 1982). Functional evidence for the cytotrophoblastic origin of the syncytial trophoblast layer 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 syncytial trophoblast *in vivo* (Richart, 1961; Galton, 1962, Gerbie *et al.*, 1968). Further evidence to support this hypothesis was provided by ultrastuctural analysis of the human term placenta (Carter, 1964; Metz *et al.*, 1979; Metz and Weihe, 1980). These studies demonstrated the presence of intercellular junctions within the syncytial trophoblast layer (Carter, 1964; Metz *et al.*, 1979) and at areas of direct contact between the villous cytotrophoblasts and the syncytial trophoblast in human chorionic villi (Metz and Weihe, 1980). The junctional complexes present within the syncytial trophoblast have been interpreted as being remnants of cytotrophoblastic junctions that have incorporated into the syncytial trophoblast cytoplasm following a cellular fusion event. To date, the cellular mechanisms that mediate the formation of the syncytial trophoblast from the underlying villous cytotrophoblasts remain poorly characterized.

The ultrastructural and functional properties of villous cytotrophoblasts and the multinucleated syncytial trophoblast differ. For example, villous cytotrophoblasts contain relatively large mitochondria and numerous free ribosomes but few rough endoplasmic reticular structures (Boyd and Hamilton, 1970). In contrast, the syncytial trophoblast contains larger nuclei and demonstrates a more extensive development of organelles associated with protein synthesis (rough endoplasmic reticulum), secretion (Golgi apparatus and secretory vesicles), and

steroid hormone biosynthesis (smooth endoplasmic reticulum, lipid vesicles, and mitochondria; Boyd and Hamilton, 1970). Despite these ultrastructural differences, villous cytotrophoblasts appear to be the primary site of synthesis of several peptides hormones, including inhibin, activin, and gonadotropin-releasing hormone (Khodr and Siler-Khodr, 1980; Miyake *et al.*, 1982; Petraglia *et al.*, 1991; 1996). Although these mononucleate trophoblastic cells can also produce human chorionic gonadotropin (hCG) at the earliest stages of pregnancy (Ohlsson *et al.*, 1989), the syncytial trophoblast becomes the major source of this and most other peptide and steroid hormones produced by the placenta throughout gestation (Hoshina *et al.*, 1982; Ringler and Strauss, 1990). These two trophoblastic cell subpopulations also demonstrate differences in their ability to transport nutrients to the fetus (Kennedy *et al.*, 1992; Furesz *et al.*, 1993) and in their susceptibility to viral infection during human pregnancy (MacCalman *et al.*, 1996b; Burton and Watson, 1997; Hemmings *et al.*, 1998).

Cytotrophoblasts at regions adjacent to the maternal tissue enter either of two distinct pathways of cellular differentiation: 1) the villous pathway in which these cells undergo terminal differentiation and fusion to form syncytium as described above or 2) the invasive extravillous pathway (Morrish *et al.*, 1998). Human extravillous cytotrophoblasts undergo extensive proliferation and breach the syncytial trophoblast layer, forming large cellular columns that extend into the maternal decidua and attach the placenta to the uterine wall during pregnancy (Enders, 1968; Muhlhauser *et al.*, 1993). Subpopulation(s) of these extravillous cytotrophoblasts subsequently dissociate from the tips of this cellular column and invade deeply into the underlying maternal tissue (Pijnenborg *et al.*, 1980). These extravillous cytotrophoblasts invade the uterine stroma and superficial myometrium as individual mononucleate cells and penetrate the basal lamina of the uterine vasculature. This cellular event is believed to result in the remodeling of the endothelial and smooth muscle cells in these blood vessels, thereby increasing blood flow to the placenta and ensuring an adequate supply of nutrients and oxygen to the

growing fetus (Brosens *et al.*, 1967; Pijnenborg *et al.*, 1983). In addition, large multinucleated trophoblastic cells, referred to as placental bed giant cells, are present in the decidua and myometrium and are most numerous at a time period that correlates with the phase of extravillous cytotrophoblast invasion (Pijnenborg *et al.*, 1981; Al-Lamki *et al.*, 1999).

The cellular mechanism(s) involved in placental bed giant cell formation are poorly understood. Some investigators have suggested that these cells are derived from the fusion of maternal decidual cells (Wynn, 1967) or from remnants of the syncytial trophoblast layer of the human placenta (Robertson and Warner, 1974). The extravillous cytotrophoblastic origin of placental bed giant cells has been supported by several studies (Pijnenborg *et al.*, 1981; Graham *et al.*, 1992; Al-Lamki *et al.*, 1999). Recent histological studies have demonstrated large aggregates of mononucleate extravillous cytotrophoblasts in direct contact with placental bed giant cells and the surrounding decidual cells, suggesting that these cells are formed by a process of cellular fusion rather than endomitosis (Al-Lamki *et al.*, 1999). This hypothesis has been supported by the observation that DNA synthesis is absent in these multinucleated placental bed giant cells, as determined by $^{3}$H-thymidine incorporation or immunoreactivity to cellular proliferation markers such as proliferating cell nuclear antigen (PCNA) or the nuclear cell proliferation-associated epitope, Ki67 (Kaufmann and Castellucci, 1997).

At the end of the first trimester of pregnancy, the basic structure of the human placenta is established and all the different trophoblastic cell subpopulations are present at the maternal-fetal interface (Boyd and Hamilton, 1970). Placental development beyond this time period involves continued trophoblastic cell proliferation and differentiation which leads to the subsequent growth and expansion of this organ until the end of the gestational period (Simpson *et al.*, 1992). The placenta, along with the vast majority of trophoblastic cells, is normally expelled from the mother following the delivery of the fetus at term.

*1.2.2: In vitro models of trophoblast differentiation*

Progress in our understanding of human trophoblast differentiation has been limited by the fact that *in vivo* human experimentation is not ethically feasible and the morphological differences that exist between the human placenta and that of experimental and domestic animals (Leiser and Kaufmann, 1994). Consequently, most of our information regarding this developmental process has relied on histological studies of post-implantation hysterectomy and term placental specimens (Hertig *et al.*, 1956; Boyd and Hamilton, 1970; Pijnenborg *et al.*, 1980). More recently, several *in vitro* model systems have been developed and used to examine specific aspects of human trophoblast differentiation (Frank *et al.*, 2000; King *et al.*, 2000).

*1.2.2-A: Villous cytotrophoblasts isolated from human term placentae*

Human term placental tissues can be enzymatically dispersed and the trophoblastic cells purified by using either density gradients or immunoselection methods. These two methods result in the isolation of highly purified populations of mononucleate cytotrophoblasts (Kliman *et al.*, 1987; Yui *et al.*, 1994; Morrish *et al.*, 1997). Villous cytotrophoblasts isolated from human term placentae enter a program of cellular differentiation that mimics many of the cellular events associated with chorionic villous formation *in vivo* (Fig. 1). In particular, freshly isolated mononucleate cytotrophoblasts undergo aggregation, differentiation, and fusion to form multinucleated syncytial structures with time in culture. The formation of multinucleated syncytium in these primary cell cultures is associated with an increase in the secretion of βhCG, a peptide hormone whose expression correlates with trophoblast differentiation and fusion *in vivo* (Hoshina *et al.*, 1982).

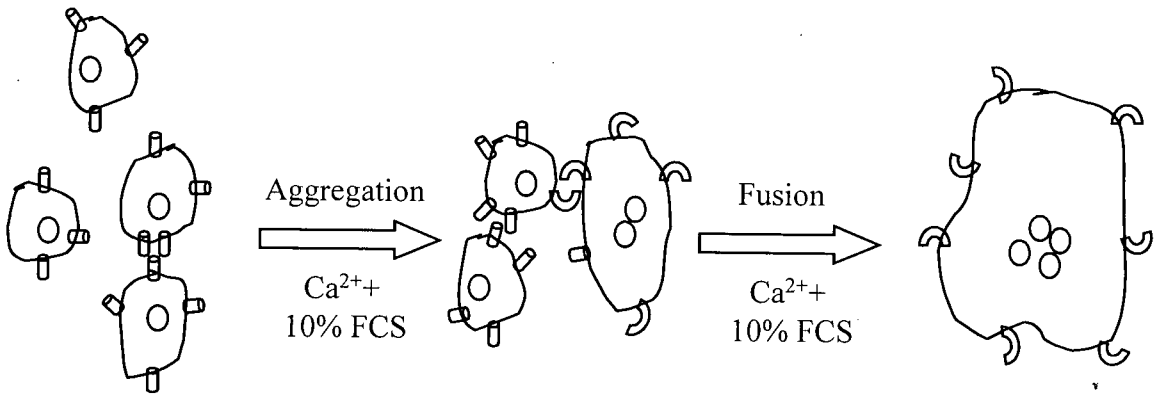



Fig 1.1: Schematic representation of human trophoblastic cell differentiation and fusion *in vitro*. Villous cytotrophoblasts isolated from human term placentae are mononucleate at early stages of culture. In the presence of 10% fetal calf serum (FCS) and $Ca^{2+}$, these cells undergo aggregation and fusion to form multinucleated syncytial structures in a time-dependent manner. The multinucleated syncytia are the predominant cellular structures present in these primary cultures at later time points.

The initial characterization of these trophoblastic cell cultures by Kliman *et al.* (1987) provided compelling evidence for the cytotrophoblastic origin of the syncytial trophoblast in the human placenta. To date, the cellular mechanisms that mediate the formation of multinucleated syncytium from mononucleate trophoblastic cells remains poorly understood.

*1.2.2-B: Transformed choriocarcinoma cell lines*

Trophoblastic cell lines derived from choriocarcinoma cells have provided a useful alternative to investigate the cell biology of human trophoblast differentiation *in vitro* (King *et al.*, 2000). Choriocarcinoma is a relatively rare malignant tumour of the human placenta that is comprised of mitotically active cytotrophoblasts (Benirschke and Kaufmann, 2000). Several choriocarcinoma cell lines have been established that exhibit varying degrees of differentiation in culture, two of which will be discussed in more detail below: BeWo and JEG-3 choriocarcinoma cells.

Patillo *et al.* (1968a; 1968b) established the first hormone producing choriocarcinoma cell line, BeWo cells. Several clones of BeWo cells have since been produced (van der Ende *et al.*, 1987; Wice *et al.*, 1990). These mononucleate trophoblastic cells share many of the cellular characteristics of cytotrophoblasts and demonstrate a low potential for differentiation and fusion *in vitro*. However, the treatment of the b30 clone of BeWo choriocarcinoma cells with forskolin, an activator of intracellular cyclic AMP (cAMP) levels (Seamon *et al.*, 1981), or 8-bromo-cAMP, a membrane-permeable cAMP analog (Tindall and Means, 1976), results in the morphological and biochemical differentiation of these cells with time in culture (Wice *et al.*, 1990; Coutifaris *et al.*, 1991). For example, cAMP treatment results in a reduction in cellular proliferation, an increase in βhCG synthesis and secretion, and the formation of large

multinucleated syncytial structures in these cultures. BeWo choriocarcinoma cells have thus been used to examine trophoblastic cell differentiation and fusion *in vitro*.

JEG-3 choriocarcinoma cells are mononucleate trophoblastic cells that were established from choriocarcinoma explant cultures by Kohler *et al.* (1971). Although an increase in intracellular cAMP levels has been shown to increase the production and secretion of βhCG in these cultures, these cells do not form multinucleated syncytium under these culture conditions (Chou *et al.*, 1978; Burnside *et al.*, 1985; Coutifaris *et al.*, 1991). Consequently, JEG-3 cells have been used as an *in vitro* model system to examine mononucleate trophoblastic cell biology.

*1.2.2-C: Extravillous cytotrophoblasts propagated from human first trimester chorionic villous explants*

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

The addition of specific growth factors to the culture medium has been shown to regulate differentiation and invasion in these primary cell cultures (Lala and Hamilton, 1996). For example, transforming growth factor-β1 (TGF-β1), which is produced by the placenta and

decidua *in vivo* (Graham *et al.*, 1992; Lysiak *et al.*, 1995), is capable of reducing proliferation and promoting the differentiation and fusion of these isolated extravillous cytotrophoblasts *in vitro* (Graham *et al.*, 1992; 1994). The formation of multinucleated cells in these primary cultures is believed to mimic the cellular events associated with placental bed giant cell differentiation *in vivo*. This terminal differentiation process has also been associated with a reduction in the invasive capacity of these isolated extravillous cytotrophoblasts (Graham and Lala, 1991). To date, the cellular mechanisms by which TGF-β1 regulates extravillous cytotrophoblastic differentiation and invasion remain poorly characterized.

## 1.3: Cellular mechanisms involved in trophoblast differentiation and invasion

The onset of trophoblastic cell differentiation along the villous or extravillous pathway is associated with changes in cell-ECM and cell-cell interactions (Burrows *et al.*, 1996; MacCalman *et al.*, 1998). For example, villous cytotrophoblasts anchored to the chorionic villous basement membrane must alter their cellular contacts with adjacent cytotrophoblasts and the underlying ECM in order to undergo terminal differentiation and fusion to form the syncytial trophoblast layer. Similarly, these cellular interactions are tightly regulated as the cytotrophoblasts enter the invasive extravillous pathway and encounter the diverse cell subpopulations and ECM components which comprise the maternal tissues encountered during the first trimester of pregnancy.

### *1.3.1: Extracellular matrix deposition*

The ECM plays a key role in maintaining tissue integrity and modulating cellular differentiation during development (Lin and Bissell, 1991; Adams and Watt, 1993). Villous

cytotrophoblasts form a polarized epithelial monolayer that is anchored to the underlying chorionic villous basement membrane. In humans, this basement membrane separates the trophoblastic cells from the villous mesenchyme and is comprised of several ECM components, including heparan sulfate protoeglycans, collagen type IV, and laminin (Ln; Earl *et al.*, 1990; Damsky *et al.*, 1992; Onodera *et al.*, 1997). Several conflicting reports exist regarding the expression of fibronectin (Fn) in the villous basement membrane, which may be partially explained by the different epitope-specific antibodies used to immunolocalize this glycoprotein or the different stages of gestation examined in these studies (Yamada *et al.*, 1987; Virtanen *et al.*, 1988; Earl *et al.*, 1990; Damsky *et al.*, 1992). However, Fn is expressed in the villous mesenchymal core at all stages of gestation examined to date.

The composition of the mesenchymal ECM is not homogeneous throughout the chorionic villi of the human placenta. For example, tenascin, an ECM glycoprotein that exhibits anti-adhesive properties *in vitro* (Aufderheide and Ekblom, 1988), is expressed at sites of cytotrophoblastic cell proliferation and in regions below degenerating syncytium (Castelluci *et al.*, 1991; Damsky *et al.*, 1992). This has led to the proposal that tenascin modulates villous cytotrophoblast differentiation. It remains unclear whether tenascin plays a direct role in promoting trophoblast differentiation along the villous or extravillous pathway or is a by-product of the cellular proliferation and differentiation associated with these developmental processes.

The extravillous cytotrophoblast column contains an ECM that is, at least in part, of trophoblastic origin and is comprised of ECM components that are typically associated with the basement membrane (Damsky *et al.*, 1992; Huppertz *et al.*, 1996; 1998; Rhode *et al.*, 1998). In addition, oncofetal Fn, an alternatively spliced variant of Fn with a unique glycopeptide epitope (Matsuura and Hakomori, 1985; Matsuura *et al.*, 1989), has been detected at sites of direct contact between this cellular column and the maternal decidua (Feinberg *et al.*, 1991). Oncofetal Fn is synthesized and secreted by trophoblastic cells in culture (Feinberg *et al.*, 1991) and

modulates cytotrophoblastic column formation *in vitro* (Aplin *et al.*, 1999). Collectively, these observations have led to the proposal that oncofetal Fn maintains placental-uterine interactions during pregnancy. The invasive extravillous cytotrophoblasts that have detached from the cellular column must then interact with the ECM of the maternal stroma which is comprised of collagen type I, III, V and Fn as well as the basement membrane-like matrix that surrounds individual decidual cells (Wewer *et al.*, 1985; Aplin *et al.*, 1988; Aplin *et al.*, 1995).

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

### *1.3.2: Extracellular matrix degradation: proteinases and their inhibitors*

Trophoblastic cells are actively involved in remodeling the ECM and have been shown to adopt similar cellular mechanisms for ECM degradation to those observed during tumour cell invasion (Yagel *et al.*, 1988b; Strickland and Richards, 1992 Lala and Hamilton, 1996). Consequently, proteinases and their associated inhibitors are believed to play a key role in

human implantation. Two major classes of proteinases have been best studied during trophoblast differentiation and invasion: plasminogen activators and matrix metalloproteinases.

*1.3.2-A: Plasminogen activators and their inhibitors*

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

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

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

significance of this switch in PAI subtype expression in these primary cell cultures remains to be determined.

*1.3.2-B: Matrix metalloproteinases and their inhibitors*

The matrix metalloproteinases (MMPs) are a gene family of zinc-dependent proteinases that mediate a variety of tissue remodeling processes (Woessner, 1991; Fata *et al.*, 2000). The MMPs are synthesized as latent precursors that must be cleaved following secretion in order to become activated. The activity of MMPs can be further regulated by the secretion of tissue inhibitors of MMPs (TIMPs).

Human trophoblastic cells have been shown to produce several MMPs, including MMP-1, -2, -3, -7, -9, -11, and -14 (Fisher *et al.*, 1989; Moll and Lane, 1990; Librach *et al.*, 1991; Auto-Harmainen *et al.*, 1992; Polette *et al.*, 1994; Nawrocki *et al.*, 1995; Vettraino *et al.*, 1996; Huppertz *et al.*, 1998; Hurskainen *et al.*, 1998; Sawicki *et al.*, 2000). MMP-2 (72 kDa type IV collagenase, gelatinase A) is expressed by the invasive extravillous cytotrophoblasts *in vivo* (Polette *et al.*, 1994) and is produced by extravillous cytotrophoblasts isolated from the human first trimester placenta (Graham *et al.*, 1993; Xu et al, 2000). Recent studies have demonstrated that MMP-2 activity can be activated by a multimeric protein complex containing MMP-14, also know as membrane type-MMP-1 (MT-MMP-1; Sato *et al.*, 1994; Young *et al.*, 1995). Interestingly, MT-MMP-1 is also expressed by human extravillous cytotrophoblasts *in vivo*, suggesting a potential autocrine mechanism for regulating MMP-2 activity during trophoblast invasion (Narwocki *et al.*, 1995; Hurskainen *et al.*, 1998).

MMP-9 (92 kDa type IV collagenase, gelatinase B) is expressed in human extravillous cytotrophoblasts *in vivo* and has been shown to be up-regulated during cytotrophoblast invasion *in vitro* (Fisher *et al.*, 1989). Function-perturbing antibodies specific for MMP-9 are capable of

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

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

TIMP-1, -2, and -3 are produced by human trophoblastic cells and decidual cells, suggesting an autocrine and paracrine regulation of MMP activity during human implantation (Graham and Lala, 1991; Polette *et al.*, 1994; Higuchi *et al.*, 1995; Hurskainen *et al.*, 1996; Ruck *et al.*, 1996). The ability of TIMP-1 and -2 to inhibit extravillous cytotrophoblast invasion has also been demonstrated *in vitro* (Graham and Lala, 1991; Librach *et al.*, 1991). Although TIMP-2 can serve as an inhibitor of MMP-2 activity, the recruitment of this secreted protein into a complex with MT-MMP-1 can also activate this proteinase (Strongin *et al.*, 1993; 1995; Young

*et al.*, 1995). Recent studies have demonstrated that TIMP-3 expression is up-regulated during trophoblast invasion *in vitro* (Bass *et al.*, 1997). The expression levels of MMP-9 and TIMP-3 are co-ordinately regulated in these primary cell cultures, suggesting that an intricate balance between the production of proteases and their inhibitors modulates the over-all proteolytic activity of trophoblastic cells during human implantation.

### *1.3.3: Cell-extracellular matrix interactions: the integrin gene superfamily of cell adhesion molecules*

Cell-ECM interactions are mediated by several classes of CAMs, one of the best characterized being the integrins (Hynes, 1992; Berman and Kozlova, 2000). This diverse gene superfamily of integral membrane glycoproteins is comprised of non-covalently associated heterodimeric α and β subunits that mediate $Ca^{2+}$-dependent cellular interactions. The ligand specificity of the different integrin heterodimers is determined by the particular combination of α and β subunits that is expressed on the cell surface. Integrin binding to specific ECM components not only anchors the cell to the surrounding substratum but is also believed to provide the molecular framework for cellular migration and activate signal transduction pathways (Lafrenie and Yamada, 1996)

Several integrin subunits are spatiotemporally expressed during human trophoblast differentiation (Bronson and Fusi, 1996; Coutifaris *et al.*, 1998). For example, the α6 and β4 integrin subunits have been localized to the villous cytotrophoblasts of human chorionic villi (Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993). The $\alpha_6\beta_4$ heterodimer is capable of binding to Ln and may link these mononucleate trophoblastic cells to the Ln-rich basement membrane in this tissue (Lee *et al.*, 1992). The expression levels of the $\alpha_6$ and $\beta_4$ integrin

subunits are down-regulated during the formation of the multinucleated syncytial trophoblast *in vivo* (Burrows *et al.*, 1993). Instead, the syncytial trophoblast layer has been shown to express the $\alpha_v\beta_3$ receptor for vitronectin (Smith *et al.*, 1990; Vanderpuye *et al.*, 1991) and the $\alpha_3\beta_1$ receptor for Ln (Gehlsen *et al.*, 1989; Korhonen *et al.*, 1991). To date, the functional significance of this switch in integrin subtype expression during the terminal differentiation and fusion of human villous cytotrophoblasts remains to be elucidated.

A reduction in the expression of the $\alpha_6$ and $\beta_4$ integrin subunits has also been demonstrated during trophoblast differentiation along the invasive extravillous pathway *in vivo* (Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993). Human extravillous cytotrophoblasts express a diverse repertoire of integrin subunits, including the $\alpha_1\beta_1$, $\alpha_5\beta_1$, and the $\alpha_v\beta_3$ heterodimeric receptors (Fisher *et al.*, 1989; Damsky *et al.*, 1992; Burrows *et al.*, 1993; Zhou *et al.*, 1993; Irving *et al.*, 1995; Zhou *et al.*, 1997a). Function-perturbing antibodies specific for $\alpha_1\beta_1$, a receptor for collagen type IV and Ln (Ignatius *et al.*, 1990), or $\alpha_v\beta_3$ are capable of inhibiting trophoblastic cell invasion *in vitro* (Damsky *et al.*, 1994; Zhou *et al.*, 1997a). Furthermore, an inhibition of $\alpha_v\beta_3$ function disrupts interaction between cytotrophoblasts and endothelial cells *in vitro*, suggesting that the integrins play a role in mediating both cell-cell and cell-ECM interactions during this developmental process (Thirkill and Douglas, 1999).

The role(s) of the $\alpha_5\beta_1$ integrin heterodimer, a receptor for Fn (Fogerty *et al.*, 1990), in extravillous cytotrophoblast differentiation remains controversial (Damsky *et al.*, 1994; Irving and Lala, 1995; Aplin *et al.*, 1999). Damsky *et al.* (1994) demonstrated that trophoblastic cells cultured in the presence of $\alpha_5\beta_1$ integrin blocking antibodies were highly invasive *in vitro*. In contrast, Irving and Lala (1995) found that function perturbing antibodies for the $\alpha_5\beta_1$ integrin heterodimer were capable of inhibiting the invasive capacity of primary cultures of extravillous cytotrophoblasts. Similarly, insulin-like growth factor binding protein-1 (IGFBP-1) has been

shown to interact with the $\alpha_5$ integrin subunit in human trophoblastic cells and thereby inhibit cellular invasion in these cultures (Irwin and Guidice, 1998). Finally, function-perturbing antibodies for the $\alpha_5\beta_1$ integrin subunits are capable of disrupting the organization of extravillous cytotrophoblast columns that develop in chorionic villous explant cultures (Aplin *et al.*, 1999). These conflicting findings may be attributed to differences in the cell isolation procedures and culture conditions employed in these studies. For example, Matrigel preparations contain multiple growth factors (Vukicevic *et al.*, 1992), which may influence the response of isolated trophoblastic cells to the $\alpha_5\beta_1$ integrin blocking antibodies in these studies.

Integrin binding to specific components of the ECM can activate signal transduction pathways in human trophoblastic cells (Burrows *et al.*, 1995). For example, tyrosine phosphorylation is enhanced in first trimester cytotrophoblasts cultured on Ln or Fn (Burrows *et al.*, 1995). Similarly, integrin subunit expression by isolated cytotrophoblasts correlated with the production of MMPs and oncofetal Fn by these cells (Bischof *et al.*, 1995). Collectively, these observations have led to the proposal that an elaborate equilibrium in trophoblast-ECM interactions exists that may provide the signaling environment required for regulating trophoblast differentiation and invasion. Indirect support for this hypothesis has been obtained from studies demonstrating alterations in the spatiotemporal expression of integrin subunits in the trophoblastic cells of preeclamptic pregnancies (Zhou *et al.*, 1993; 1997b). However, no difference in the expression of integrin subunits was observed in the third trimester placenta of these pathological pregnancies, when the clinical features of this disease are manifested (Divers *et al.*, 1995). It remains unclear whether the aberrant expression of integrin subunits in preeclampsia is the cause or effect of the cellular events associated with this disease of pregnancy.

*1.3.4: Cell-cell interactions*

During human implantation, trophoblastic cells must alter their repertoire of adhesion molecules as these cells undergo differentiation and invasion in the maternal endometrium (Burrows *et al.*, 1996; Kimber and Spanswick, 2000). To date, several CAMs have been identified during the initial stages of implantation. For example, trophinin is a recently characterized integral membrane glycoprotein that is expressed in the human endometrial epithelium and in the trophectoderm of monkey blastocysts (Fukada *et al.*, 1995). Trophinin is capable of interacting with two cytoplasmic proteins, tastin and bystin, and can mediate $Ca^{2+}$-dependent homophilic interactions *in vitro* (Fukada *et al.*, 1995; 1999; Suzuki *et al.*, 1998). These observations have led to the proposal that trophinin, in association with tastin and bystin, mediates trophoblast-endometrial interactions during blastocyst implantation. Although this adhesion complex has been localized to the apical membrane domain of the human syncytial trophoblast during the first trimester of pregnancy (Suzuki *et al.*, 1999), the role of trophinin in regulating trophoblastic cell interactions in the human remains unclear.

Despite the recognition that trophoblastic cells form complex homotypic and heterotypic interactions during human implantation, our understanding of the adhesive mechanisms that mediate these cellular interactions remains limited. To date, most studies have focused on the expression of different members of the immunoglobin (Ig) gene superfamily of $Ca^{2+}$-independent CAMs in human trophoblastic cells (Buck, 1992; Burrows *et al.*, 1996). In particular, extravillous cytotrophoblasts express intercellular (I)-CAM-1, neural (N)-CAM, vascular (V)-CAM-1, platelet-endothelial (PE)-CAM-1, melanoma (Mel)-CAM, and carcinoembryonic antigen (CEA)-CAM during the first trimester of pregnancy (Damsky *et al.*, 1992; Burrows *et al.*, 1994; Shih and Kurman, 1996; Coukos *et al.*, 1998; Bamberger *et al.*, 2000). These trophoblastic cells also express endothelial (E)-selectin (Milstone *et al.*, 2000), a member of the selectin gene family of $Ca^{2+}$-dependent CAMs that mediates leukocyte-endothelial cell

interactions during inflammatory responses (Vestweber, 1992; Varki, 1994). The biological significance of these expression patterns remains largely undefined. For example, Shih *et al.* (1998) have recently demonstrated that a putative ligand for Mel-CAM is present in the myometrial smooth muscle cells, suggesting that this CAM limits the invasion of extravillous cytotrophoblasts to the superficial layers of the human myometrium. Functional studies are required to complement these descriptive observations if the biological role(s) of these CAMs at the maternal-fetal interface are to be fully understood.

The establishment of cell-cell communication by the regulated expression of gap junction components, known as the connexins (Cx), has recently been investigated during human trophoblast differentiation and invasion. For example, isolated human trophoblastic cells express the Cx subtype, Cx43 (Cronier *et al.*, 1994; Khoo *et al.*, 1998). Khoo *et al.* (1998) demonstrated a marked reduction in the expression levels of Cx43 following the transformation of normal extravillous cytotrophoblast cultures with the SV40 large T antigen, suggesting a role for gap junctional communication in preventing neoplastic changes in these cells. These findings have been supported by more recent studies in which the transfection of different Cx subtypes into JEG-3 cells was capable of inhibiting cellular proliferation in this choriocarcinoma cell line (Hellmann *et al.*, 1999; Winterhager *et al.*, 2000). The role of gap junctions in the terminal differentiation and fusion of human villous cytotrophoblasts remains more contentious. Although early ultrastructural observations demonstrated the presence of gap junctions between villous cytotrophoblasts and the syncytial trophoblast in the first trimester placenta (De Viergiliis *et al.*, 1982), these junctions were not present in the trophoblastic cells of the human term placenta (Metz *et al.*, 1979; 1980). Despite this discrepancy, cytotrophoblasts isolated from term placentae express Cx43 and the levels of this membrane protein decrease as these cells aggregate and fuse to form syncytium with time in culture (Cronier *et al.*, 1994; 1999; Winterhager *et al.*,

1999). To date, the role of gap junctions in this developmental process as well as the factors that regulate their activity at the cell surface remain to be elucidated.

In view of our current understanding of the adhesive mechanisms involved in regulating trophoblast differentiation during human implantation and placentation, we have chosen to focus our studies on the gene superfamily of CAMs known as the cadherins.

### 1.4: The cadherin gene superfamily of cell adhesion molecules

The cadherins are integral membrane glycoproteins that mediate $Ca^{2+}$-dependent cell adhesion in a homophilic manner (Takeichi, 1991; 1995; Potter *et al.*, 1999). Based on amino acid sequence homology and structural features, the cadherin gene superfamily has been grouped into at least two distinct subfamilies: the classical cadherins, which can be further subdivided into type 1, type 2, and unclassified classical cadherins, and the non-classical cadherins, which can be further subdivided into truncated cadherins, desmosomal cadherins, protocadherins, and unclassified cadherin-related proteins (Suzuki, 1996; Nollet *et al.*, 2000).

Most members of the cadherin gene superfamily are type 1 integral membrane proteins that are composed of an amino terminal extracellular domain, a transmembrane domain, and a carboxy terminal cytoplasmic domain (Grunwald, 1993). The extracellular domain of the cadherins is comprised of a variable number of cadherin-repeat motifs that are approximately 110 amino acids in length (Hatta *et al.*, 1988; Mahoney *et al.*, 1991; Sano *et al.*, 1993). These repeat motifs contain several highly conserved amino acid sequences. For example, the amino acid sequences, LDRE, DXD, and DXNDN (D=aspartic acid, E=glutamic acid, L=leucine, N=asparagine, R=arginine, and X= any amino acid), are present within the cadherin repeat motifs (Hatta *et al.*, 1988). These amino acid sequences have been shown to bind $Ca^{2+}$ and stabilize the structural conformation of the cadherin extracellular domain (Ringwald *et al.*, 1987;

Nagar *et al.*, 1996; Pertz *et al.*, 1999). However, the amino acid sequences within the cadherin repeat motifs differ from one another within a cadherin protein and among the different subfamily members. Furthermore, the cytoplasmic domain of the cadherins is highly variable in amino acid length and composition among different cadherin subfamilies (Suzuki, 1996). Collectively, the number and amino acid homology of the cadherin extracellular and cytoplasmic domains has led to the classification system described below.

### *1.4.1: Type 1 classical cadherins*

#### *1.4.1-A: Characterization and structure of the type 1 classical cadherins*

The cadherins were originally identified by immunological techniques in several studies examining cell-cell interactions and the morphogenetic events associated with pre-implantation development in mouse embryos (Takeichi, 1977; Kemler *et al.*, 1977; Hyafil *et al.*, 1980). However, one of the earliest suggestions for specific cellular mechanism(s) that direct animal morphogenesis has been traced back to the studies of Townes and Holtfreter (1955; Steinberg, 1996). In these studies, the embryonic cells of several amphibian species were dissociated by alkaline treatment and allowed to reorganize under neutral pH conditions. These embryonic cells reorganized in a tissue specific manner *in vitro*. The molecular basis for this selective reorganization was subsequently identified in a series of studies examining the ability of antibodies directed against cell surface proteins to interfere with compaction in mouse pre-implantation embryos, a developmental process that is dependent on the presence of $Ca^{2+}$ and changes in cell-cell interactions (Ducibella *et al.*, 1975; Ducibella and Anderson, 1975; 1978). These inhibitory antibodies were used to purify a membrane glycoprotein, initially termed uvomorulin, from murine blastocysts (Kemler *et al.*, 1977; Hyafil *et al.*, 1980; 1981). Similar

approaches were used to identify chicken liver-CAM (L-CAM; Bertolotti *et al.*, 1980), canine Arc-1 (Imhof *et al.*, 1983; Behrens *et al.*, 1985), and human epithelial cell-CAM 120/80 (Damsky *et al.*, 1983). These molecules appear to be species-specific homologues of the murine 124 kDa proteins species first referred to as E-cadherin (E-cad) in the studies of Yoshida-Noro *et al.* (1984).

The type 1 classical cadherins include the three originally identified by immunological and cDNA cloning strategies; E-cad, N-cadherin (N-cad), P-cadherin (P-cad), as well as the more recently cloned R-cadherin (R-cad; Yoshida-Noro *et al.*, 1984; Hatta *et al.*, 1985; Nose and Takeichi, 1986; Nose *et al.*, 1987; Nagafuchi *et al.*, 1987; Hatta *et al.*, 1988; Inuzuka *et al.*, 1991a; 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 distributions during mouse embryonic development; E-cad is primarily expressed in epithelial cells, N-cad in neuronal cells, P-cad in the placenta and decidua, and R-cad in the retina.

The type 1 classical cadherins are comprised of an extracellular domain that contains four cadherin repeat motifs (EC1-EC4) and a poorly conserved membrane proximal subdomain (EC5), a single transmembrane domain, and two highly conserved cytoplasmic subdomains (Fig. 2; Grunwald, 1993). These CAMs are synthesized as precursor molecules containing a signal peptide and an amino terminal domain that is removed post-translationally by the furin/substilin 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 al.*, 1999). In addition, the EC5 subdomain of the type 1 classical cadherins contains four conserved cysteine residues that are likely involved in the formation of intramolecular disulfide bonds (Ringwald *et al.*, 1987; Takeichi, 1991).

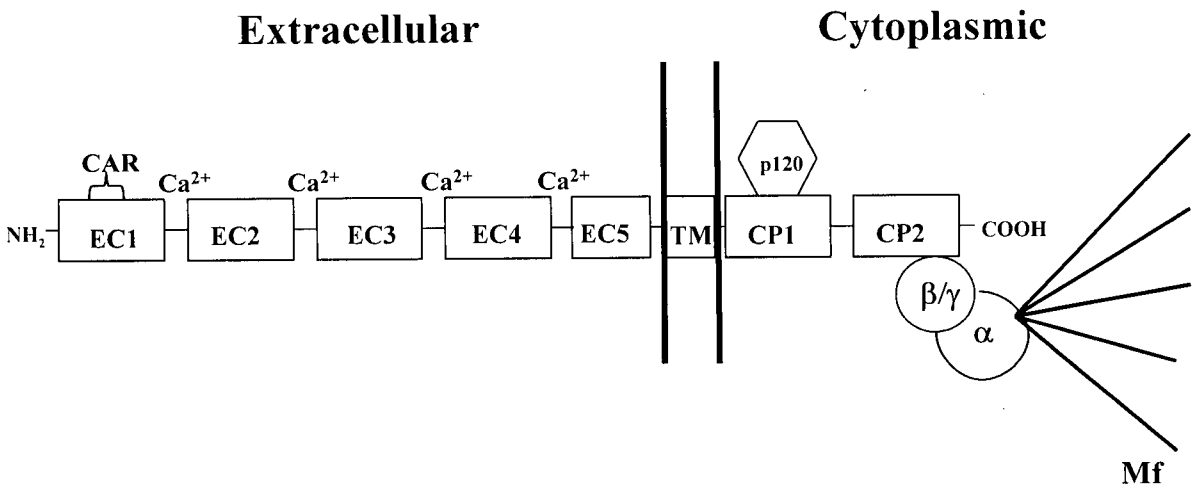



Fig 1.2: Schematic representation of the basic structure of the type 1 classical cadherins in the plasma membrane. The cadherins are comprised of five extracellular subdomains (EC1-EC5), a single transmembrane domain (TM), and two cytoplasmic subdomains (CP1 and CP2). The EC1 subdomain contains the CAR sequence, HAV, which is believed to play a role in cadherin-mediated adhesion. In addition, $Ca^{2+}$-binding sites are present at the interface between the extracellular subdomains. The cytoplasmic subdomains are the most highly conserved regions among members of the type 1 classical cadherins and interact with a family of cytoplasmic proteins known as the catenins. In particular, CP1 interacts with $p120^{ctn}$ whereas CP2 forms multimeric complexes with either β- or γ-catenin and α-catenin. These interactions are believed to link the cadherins to the actin-based microfilaments of the cytoskeleton.

The EC1 subdomain of the type 1 classical cadherin subtypes, which is the most distal domain from the plasma membrane, harbours the cell adhesion recognition (CAR) sequence, HAV (H=histidine, A=alanine, and V=valine; Blaschuk *et al.*, 1990a; 1990b). This CAR sequence is believed to contribute to the adhesive properties of these cadherin subtypes. For example, synthetic peptides generated against this amino acid sequence have been shown to inhibit cadherin-dependent processes, including neurite outgrowth, embryonic compaction, and epithelial cell differentiation (Blaschuk *et al.*, 1990a; Doherty *et al.*, 1991; Noe *et al.*, 1999). The non-conserved amino acid residues immediately adjacent to the CAR sequence are thought to modulate the ability of the type 1 classical cadherins to interact with one another in a homophilic manner (Nose *et al.*, 1990; Williams *et al.*, 2000).

Recent studies, using high resolution crystallography, have provided insight into the three-dimensional structure of the type 1 classical cadherin extracellular domains and the mechanism of homophilic interactions (Koch *et al.*, 1999; Leckband and Sivasankar, 2000). The crystal structure of the EC1 subdomain of N-cad revealed the presence of *cis* and *trans* interactions in this protein fragment (Shapiro *et al.*, 1995). In view of these observations, Shapiro *et al.* (1995) proposed a model for cadherin-mediated adhesion in which the cadherins interact with an identical subtype on the surface of the same cell and on the surface of an adjacent cell in a zipper-like fashion. Furthermore, structural analysis of the E-cad extracellular domain suggested a two-step model for cadherin-mediated adhesion in which *cis* interactions serve as a prerequisite for subsequent *trans* interactions, but provided no direct evidence for zipper like interactions (Tomschky *et al.*, 1996). Although somewhat conflicting, these structural studies have highlighted the importance of lateral interactions in regulating cadherin-mediated adhesion. These observations have been supported by several *in vitro* and *in vivo* studies examining the role of lateral clustering in regulating cadherin-based homophilic interactions (Adams *et al.*, 1996a; Brieher *et al.*, 1996; Klingelhofer *et al.*, 2000; Lambert *et al.*, 2000; Murase *et al.*, 2000).

The transmembrane domain of the type I classical cadherins is comprised of approximately 30 amino acids that are relatively hydrophobic and are believed to assume an α-helical conformation in the plane of the membrane (Hatta *et al.*, 1988; Gruwald, 1993). In addition, the cadherin transmembrane domain contains several conserved leucine residues that have been shown to contribute to the lateral clustering of E-cad at the cell surface (Gurezka *et al.*, 1999; Huber *et al.*, 2000).

The cytoplasmic domains are the most highly conserved regions among members of the type 1 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 (Chen *et al.*, 1997; Ozawa and Kemler, 1998; Yap *et al.*, 1998; Murase *et al.*, 2000). The classical cadherin cytoplasmic domains are capable of interacting with a group of cytoplasmic proteins, known as the catenins.

*1.4.1-B: Classical cadherin-catenin interactions*

Cadherin function is, at least in part, regulated by interactions with the catenins (Hirano *et al.*, 1992; Oyama *et al.*, 1994; Aberle *et al.*, 1996; Gumbiner, 2000). The catenins were first identified by immunoprecipitation studies with the type 1 classical cadherins and included α-, β-, and γ-catenin (also known as plakoglobin; Ozawa *et al.*, 1988; Ozawa and Kemler, 1992). Plakoglobin is biochemically identical to γ-catenin (Knudsen and Wheelock, 1992; Wahl *et al.*, 1996; Lewis *et al.*, 1997) and was also identified as a major component of desmosomal junctions (Cowin *et al.*, 1986). β- and γ-catenin have since been shown to bind to a serine-rich 30 amino acid sequence in the carboxy terminal cytoplasmic domain of the classical cadherins (Ozawa *et*

*al.*, 1990; Stappert and Kemler, 1994). These two cytoplasmic proteins interact with the cadherins in a mutually exclusive manner (Butz and Kemler, 1994; Nathke *et al.*, 1994).

cDNA cloning studies have revealed a high degree of amino acid homology between β- and γ-catenin (McCrea *et al.*, 1991; Butz *et al.*, 1992; Fouquet *et al.*, 1992). In particular, these two proteins share an imperfect 42 amino acid repeat motif, referred to as an armadillo (*arm*) motif, that was first identified in the *Drosophila* segment polarity gene, *arm* (Riggleman *et al.*, 1989; Peifer, and Wieschaus, 1990; Hatzfield 1999). The binding of both β- and γ-catenin to the cadherin cytoplasmic domain is mediated, at least in part, by amino acid residues that are contained in the 12 or 13 *arm* repeats present in the respective proteins (Hulsken *et al.*, 1994; Aberle *et al.*, 1994; Sacco *et al.*, 1995; Ozawa *et al.*, 1995; Troyanovsky *et al.*, 1996; Wahl *et al.*, 1996). Recent high resolution crystallography studies of β-catenin have provided further evidence for the importance of this motif in mediating interactions with the cadherins (Huber *et al.*, 1997). Moreover, the binding of β-catenin to the cytoplasmic domain of the type 1 classical cadherins alters the structural conformation of the cadherin carboxy tail, suggesting a key role for this catenin subtype in regulating the function of this CAM at the cell surface (Huber *et al.*, in the press).

Although β- and γ-catenin exhibit a high degree of structural similarity and bind the same region of the type 1 classical cadherin cytoplasmic domain, these two cellular proteins also demonstrate discrete functional properties (Ben-Ze'ev and Geiger, 1998; Zhurinsky *et al.*, 2000). For example, β-catenin interacts with several proteins at the cell surface, including the tight junction associated protein, ZO-1 (Rajaseharan *et al.*, 1996), the multi-functional protein, presenilin-1 (Yu *et al.*, 1998; Georgakopolous *et al.*, 1999), and the scaffolding PDZ domain-containing protein, LIN-7 (Perego *et al.*, 2000). In contrast, γ-catenin can bind directly to desmoglein (Marthur *et al.*, 1994; Troyanovsky *et al.*, 1994b), desmocollin (Troyanovsky *et al.*,

1994b), and desmoplakin (Kowalczyk *et al.*, 1997), three proteins that are localized to desmosomal junctions in epithelial cells. Furthermore, γ-catenin could not be substituted for β-catenin during the early embryonic development of β-catenin null mutant mice (Haegel *et al.*, 1996; Huelsken *et al.*, 2000). Homozygous -/- β-catenin null mutant mice fail to survive beyond the early stages of gastrulation and demonstrate severe defects in anterior-posterior axis formation. In contrast, γ-catenin gene knock-out mice survive until day 16 of embryogenesis and subsequently die as a result of heart and skin defects (Bierkamp *et al.*, 1996; 1999; Ruiz *et al.*, 1996). Taken together, these observations suggest that the two catenin subtypes mediate the formation of structurally and functionally distinct cadherin-catenin complexes at the cell surface.

α-catenin is believed to link the cadherin-catenin complex to the actin-based cytoskeleton (Ozawa *et al.*, 1990). This cytoplasmic protein has been shown to bind directly to both β- and γ-catenin (Huber *et al.*, 1997; Koslov *et al.*, 1997; Nieset *et al.*, 1997; Obama and Ozawa, 1997). α-catenin exhibits a high degree of amino acid homology with the actin-binding protein, vinculin (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991; Menkel *et al.*, 1994; Johnson and Craig, 1994), and is also capable of binding to actin (Rimm *et al.*, 1995a). These observations have led to the proposal that α-catenin bridges the cadherin-catenin complex directly to the cytoskeleton. The linkage of the cadherin-catenin complex to the actin-based microfilaments is believed to strengthen cadherin-mediated interactions, at least in cultured epithelial cells (Adams *et al.*, 1996b; Vasiouhkin *et al.*, 2000).

α-catenin can interact with multiple actin-binding proteins, including α-actinin (Knudsen *et al.*, 1995), vinculin (Weiss *et al.*, 1998), and myosin (Kussel-Andermann *et al.*, 2000), suggesting that binding to the microfilaments may not be mediated directly by this catenin subtype (Provost and Rimm, 1999). α-catenin may also link the cadherin-catenin complexes to the microtubules of the cytoskeleton (Kaufmann *et al.*, 1999; Chausovsky *et al.*, 2000). Although

the precise mechanism by which α-catenin mediates these interactions has not yet been determined, several studies have demonstrated a critical role for α-catenin in regulating cadherin-mediated adhesion (Hirano *et al.*, 1992; Shimoyama *et al.*, 1992; Ewing *et al.*, 1995; Torres *et al.*, 1997). For example, α-catenin gene mutant mice fail to form a functional trophectodermal layer, a developmental process that is dependent on E-cad-mediated adhesion (Hyafil *et al.*, 1980; 1981; Torres *et al.*, 1997). α-catenin is also capable of interacting with ZO-1 (Itoh *et al.*, 1997) and spectrin (Pradhan *et al.*, in the press), but the role(s) of these multimeric protein complexes in regulating cadherin-mediated adhesion remain poorly understood.

$p120^{ctn}$, a cytoplasmic protein that is distantly related to β- and γ-catenin (Reynolds *et al.*, 1992; Peifer *et al.*, 1994a), has recently been identified as a component of these distinct cadherin-catenin complexes (Reynolds *et al.*, 1994; Shibamato *et al.*, 1995). cDNA cloning studies have revealed that $p120^{ctn}$ is comprised of 10 *arm* repeat motifs which are involved in the interaction between this catenin subtype and the type 1 classical cadherin cytoplasmic domain (Reynolds *et al.*, 1992; 1996). $p120^{ctn}$ binds to a highly conserved region of the cadherin cytoplasmic domain that is more proximal to the plasma membrane than the carboxy terminal β- and γ-catenin binding region (Ozawa and Kemler, 1998; Yap *et al.*, 1998; Ohkubo and Ozawa, 1999). In addition, $p120^{ctn}$ is not present in α-catenin immunoprecipitated complexes and is therefore not believed to link the cadherins to the cytoskeleton (Daniel and Reynolds, 1995). The state of phosphorylation of $p120^{ctn}$ appears to influence the adhesive activity of E-cad by modulating the ability of this CAM to form lateral interactions at the cell surface (Aono *et al.*, 1999; Ohkubo and Ozawa, 1999). As $p120^{ctn}$ was first identified as a major substrate for the *src* tyrosine kinases (Reynolds *et al.*, 1989) and the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony stimulating factor-1 (CSF-1) receptor tyrosine kinases (Downing and Reynolds, 1991), it has been suggested that this cytoplasmic protein plays a key

role in regulating cadherin-mediated adhesion and may be involved in the intracellular signaling pathways activated by the distinct cadherin-catenin complexes.

In addition to their role(s) in regulating cadherin-mediated adhesion and linkage to the cytoskeleton, the catenins have been shown to be integral components of signal transduction pathways (Gumbiner, 1995; Daniel and Reynolds, 1997; Ben-Ze'ev *et al.*, 2000). As β-catenin is the mammalian homologue of the *Drosophila arm* gene (McCrea *et al.*, 1991), a known component of the wnt signaling pathway that determines body axis formation in *Xenopus* embryos (Dale 1998; Willert and Nuse, 1998), most studies have focused on the role(s) of this catenin subtype in intracellular signaling processes. Activation of the wnt pathway results in an increase in cytosolic β-catenin levels and the nuclear translocation of a multimeric complex that contains this catenin subtype and the Lymphocyte Enhancing Factor/T cell Factor (LEF/TCF) family of transcription factors (Behrens *et al.*, 1996; Huber *et al.*, 1996; Papkoff *et al.*, 1996). Although this complex has been shown to influence the transcription of target genes in a variety of systems (Korinek *et al.*, 1997; Morin *et al.*, 1997; Simcha *et al.*, 1998), the formation and translocation of β-catenin/LEF-1 complexes is not sufficient to activate gene expression, per se (Prieve and Waterman, 1999).

The ability of β-catenin to participate in signal transduction is believed to be regulated by interactions with several cytoplasmic and membrane-associated proteins. For example, interactions with glycogen synthase kinase 3β, a target of the wnt signaling pathway, the adenomatous polyposis coli (APC) protein, a gene product that is frequently mutated in human colorectal carcinoma, and the scaffolding proteins axin or conductin, regulate the degradation of β-catenin by the ubiquitin-proteasome pathway (Su *et al.*, 1993; Aberle *et al.*, 1997; Behrens *et al.*, 1998; Ikeda *et al.*, 1998; Easwaran *et al.*, 1999). In addition, β-catenin interactions with either the cadherin cytoplasmic domain or α-catenin restrict the localization of β-catenin to the

plasma membrane, thereby inhibiting its ability to activate transcription in cultured epithelial cells (Sadot *et al.*, 1998; Simcha *et al.*, 1998). In contrast, the overexpression of γ-catenin in these cells not only results in the translocation of β-catenin/LEF-1 complexes to the nucleus and transcriptional activation of reporter gene constructs (Simcha *et al.*, 1998), but also increases the degradation of β-catenin by the ubiquitin-proteasome system (Salomon *et al.*, 1997). Finally, β-catenin mediates the interaction of the cadherin-catenin complex with the EGF receptor (Hoschuetzky *et al.*, 1994) and the IGF type I receptor (Playford *et al.*, 2000), directly linking cadherin function with receptor-mediated signaling. The activation of these receptors results in the phosphorylation of β-catenin on tyrosine residues and the disruption of E-cad-mediated adhesion (Hazan and Norton, 1998; Playford *et al.*, 2000). Taken together, these observations suggest that the β-catenin binding to the cadherin cytoplasmic domain modulates the ability of this cytoplasmic protein to regulate adhesion, transcription, or enter a pathway of degradation.

*1.4.1-C: Cell biology of the type 1 classical cadherins*

The type 1 classical cadherins are believed to be key morphoregulators during embryonic development and maintain tissue integrity in adults (Takeichi; 1991; 1995; Gumbiner; 1996). The spatiotemporal expression of these cadherin subtypes is tightly regulated during developmental processes such as cell migration, cell rearrangements and sorting, epithelial-mesenchymal transitions, tissue remodeling, cell differentiation, and organogenesis. For example, E-cad is expressed in both unfertilized and fertilized oocytes, although the expression of this CAM at this stage of development results from the translation of stored maternal E-cad mRNA transcripts (Vestweber *et al.*, 1987; Sefton *et al.*, 1992; Ohsugi *et al.*, 1996). The transition from maternal to embryonic expression occurs between the two to four cell stage in

mouse embryos, but it is not until the late eight cell stage that E-cad is localized to areas of cell-cell contact (Vestweber *et al.*, 1987). In contrast, P-cad is first expressed in the mural trophectoderm of murine blastocysts and in the decidua following implantation (Nose and Takeichi, 1986; Kadokawa *et al.*, 1989). N-cad is first detected in the embryonic mesodermal cells during the early stages of gastrulation (Hatta and Takeichi, 1986; Hatta *et al.*, 1987), whereas R-cad is only expressed at later stages of organogenesis in several tissues including the eye, brain, and skeletal muscle (Inuzaki *et al.*, 1991a; 1991b; Matsunami and Takeichi, 1995; Rosenberg *et al.*, 1997). The type 1 classical cadherins are expressed at high levels in most adult tissues. In general, these tissue distributions are maintained in the human. One major exception is that P-cad is not expressed in the human placenta or decidua (Shimoyama *et al.*, 1989; 1991).

Embryonic cells displaying different type 1 classical cadherin subtypes segregate from one another and it is believed that the regulated expression of these CAMs provides the molecular basis for the segregation and sorting of discrete cell populations and the subsequent formation of tissues during development. For example, progenitor cells in the embryonic ectoderm that are destined to differentiate into the neural tube switch from expressing E-cad to N-cad in mouse and chicken embryos (Thiery *et al.*, 1984; Hatta and Takeichi, 1986; Hatta *et al.*, 1987). The switch in cadherin subtype expression correlates with the separation of the cells that are expressing N-cad from the E-cad-expressing ectodermal cell layer and the subsequent organization of the neural tube in these embryos. The role of cadherin-mediated homophilic interactions in cell sorting was first demonstrated in a series of studies examining the effects of transfecting a full-length E-cad or N-cad cDNA into fibroblastic L cells, (Nagafuchi *et al.*, 1987; Hatta *et al.*, 1988; Nose *et al.*, 1988). These cells underwent $Ca^{2+}$-dependent aggregation following transfection and preferentially adhered to adjacent cells expressing an identical cadherin subtype. A more recent study by Steinberg and Takeichi (1994) has demonstrated that

cell sorting is dependent not only on the type 1 classical cadherin expressed by the transfected L cells but also on the level of cadherin subtype expression.

The relative importance of the type 1 classical cadherins in regulating morphogenesis has been highlighted by recent gene knock-out studies (Hynes, 1996). For example, a null mutation for the E-cad gene results in embryonic lethality in the mouse (Larue *et al.*, 1994; Reithmacher *et al.*, 1995). E-cad null mutant embryos undergo compaction but subsequently fail to form a functional trophectodermal layer and die prior to implantation. The ability of these mutant mice to undergo compaction has been explained by these authors as the result of maternally derived E-cad mRNA transcripts. In contrast, N-cad gene knock-out mice exhibit severe cardiac defects and die by day 10 of gestation (Radice *et al.*, 1997a). Although N-cad is believed to play a key role in neural tube formation, these mutant mice are capable of forming this tissue structure. However, neural defects and aberrant somitogenesis become apparent at later stages of gestation in these N-cad-deficient mice (Radice *et al.*, 1997a; Linask *et al.*, 1998). P-cad gene knock-out mice survive until term and are fertile as adults but females demonstrate aberrant mammary gland development later in life (Radice *et al.*, 1997b). This result is somewhat surprising given the high levels of P-cad expression in the mouse placenta and decidua and its putative role in mediating placental-uterine interactions (Kadokawa *et al.*, 1989). These observations suggest that other cadherin subtypes are capable of compensating for the loss of N- and P-cad function in the respective mutant mouse embryos.

The type 1 classical cadherins govern the developmental fate of cells. In particular, murine embryonic stem cells lacking endogenous cadherin expression enter discrete programs of differentiation following the transfection of different cadherin subtypes (Larue *et al.*, 1996; Rosenberg *et al.*, 1997). For example, the transfection of a full-length E-cad cDNA into these embryonic stem cells results in a reduction in the mRNA levels encoding mesoderm-specific transcription factors, such as T-brachyury (Herrmann, 1991; Larue *et al.*, 1996). These stem cells

exclusively formed epithelial cell structures when injected into syngeneic mice. In contrast, the transfection of a full-length N-cad cDNA into the same cell type had no effect on the levels of the mRNA transcript that encode for T-brachyury, and neuoroepithelial structures and cartilage were formed in tumours derived from these cell lines. Exogenous R-cad expression in these embryonic stem cells resulted in striated muscle formation (Rosenberg *et al.*, 1997). In contrast, the ability of P-cad to influence tissue formation in this model system has yet to be determined.

The mechanism(s) by which these cadherin subtypes modulate cellular differentiation remain poorly understood. However, recent studies have shown that E-cad-mediated adhesion is capable of regulating the expression of different integrin subunits in isolated human epidermal keratinocytes (Hodivala and Watt, 1994; Sastry and Horowitz, 1996; Zu and Watt; 1996). Similarly, ectopic N-cad expression in primary cultures of quail myoblasts regulates contact inhibition and motility in these cultures, at least in part, by an induction of integrin expression (Huttenlocher *et al.*, 1998). A recent study has demonstrated that a nonreceptor tyrosine kinase, Fer, is capable of mediating cross-talk between N-cad and the β1 integrin subunit during chick neural retina explant outgrowth (Arregui *et al.*, 2000). Taken together, these observations suggest that the type 1 classical cadherins may modulate the repertoire of adhesion receptors expressed on the cell surface and thereby play integral role(s) in cellular differentiation.

The type 1 classical cadherins mediate the formation of tight, gap, and desmosomal junctions before they are localized to the adherens junctions in well-differentiated epithelial cells (Angres *et al.*, 1996; Adams *et al.*, 1996). Epithelial intercellular junctional complexes include tight junctions, adherens junctions, desmosomes, and gap junctions (Farquhar and Palade, 1963). To date, most studies have focused on the effects of inhibiting cadherin function on junctional complex assembly (Gumbiner *et al.*, 1988; Wheelock and Jenson, 1992; Troxell *et al.*, 1999; 2000). For example, function-perturbing antibodies specific for E-cad disrupt the formation of gap junctions, desmosomes, and tight junctions in epidermal keratinocytes (Wheelock and

Jensen, 1992) and Madine Darby canine kidney (MDCK) cells (Gumbiner *et al.*, 1988), an *in vitro* model for polarized epithelial cells (Simmons, 1982). However, the inhibition of cadherin function in MDCK cells using a dominant negative mutant E-cad cDNA construct lacking a complete extracellular domain resulted in an increase in tight junction assembly (Troxell *et al.*, 2000). These differences might be explained by the different techniques employed to inhibit endogenous cadherin function or the presence of endogenous cadherin subtypes in MDCK cells that had not previously been detected (Stewart *et al.*, 2000). Although the transfection of a full-length E-cad cDNA into retinal pigment cells mediates the formation of desmosomes in these cells (Marrs *et al.*, 1995), this CAM may not be required for the absolute maintenance of these junctions as the blastomeres of E-cad-deficient embryos are also capable of forming desmosomes (Riethmacher *et al.*, 1995). The importance of E-cad in maintaining epithelial cell differentiation, however, is highlighted by the failure of these cells to form a polarized epithelium despite the presence of these desmosomal junctions in these null-mutant embryos.

E-cad is involved in the establishment of epithelial cell polarity (Yap *et al.*, 1997; Braga, 2000). Newly synthesized E-cad is targeted specifically to the basolateral membrane domain of epithelial cells and is actively internalized and recycled to this membrane domain, suggesting a role for this CAM in the trafficking and targeting of membrane components to the basolateral membrane surface (Le Bivic *et al.*, 1990; Chen *et al.*, 1999; Lee *et al.*, 1999). In addition, E-cad has been shown to restrict the localization of the sodium, potassium-adenosine triphosphatase ($Na^+$, $K^+$-ATPase) to the basolateral membrane domain of a variety of polarized epithelial cells, including mural trophectodermal cells, MDCK cells, and thyroid epithelial cells (Watson *et al.*, 1990; Yap *et al.*, 1995; Pipenhagen and Nelson, 1998). For example, the transfection of a full-length E-cad cDNA into fibroblastic cells and retinal epithelial cells was capable of localizing the $Na^+$, $K^+$-ATPase to the basolateral membrane in these cell cultures (McNeill *et al.*, 1990; Marrs *et al.*, 1995). The interaction between E-cad and the $Na^+$, $K^+$-ATPase may, at least in part,

explain why E-cad deficient embryos fail to form a blastocoele cavity *in vivo* (Larue *et al.*, 1994). In thyroid epithelial cell cultures, E-cad is first localized to areas of cell-cell contact and is subsequently restricted to the basolateral membrane domain along with the $Na^+$, $K^+$-ATPase as these cells become polarized (Yap *et al.*, 1995). Collectively, these observations suggest that E-cad mediates an early adhesive event that serves as a prerequisite for the recruitment and organization of membrane components that are involved in maintaining the differentiated epithelial cell state.

E-cad, in association with the catenins, plays a key role in regulating the non-invasive phenotype of epithelial cells *in vitro* and *in vivo* (Birchmeier and Behrens, 1994; Mareel *et al.*, 1996). Early studies demonstrated that the inhibition of E-cad-mediated interactions, using function-perturbing antibodies, were capable of altering the morphology and promoting the ability of MDCK cells to invade collagen gels and embryonic chicken heart tissue explants *in vitro* (Behrens *et al.*, 1989). Furthermore, the transfection of a full-length E-cad cDNA into invasive carcinoma cells not only decreased the invasive potential of these cells, but also induced a reversion to the polarized epithelial cell state (Frixen *et al.*, 1991). More recently, ectopic E-cad expression in prostate carcinoma cells was capable of inhibiting MMP-2 activity suggesting a further mechanism by which E-cad may modulate cellular invasion (Luo *et al.*, 1999). Moreover, the human E-cad gene is the target of transcription factors such as Snail (Battle *et al.*, 2000; Cano *et al.*, 2000) and WT1 (Hosono *et al.*, 2000) which are capable of either promoting or inhibiting cellular invasion by differentially regulating E-cad expression in carcinoma cells and fibroblastic cells, respectively. Several studies have demonstrated an inverse correlation between the levels of E-cad expression and the invasive capacity of human carcinoma cells *in vivo* (Birchmeier *et al.*, 1991; Potter *et al.*, 1999). These observations have led to the hypothesis that E-cad is the product of a tumour suppressor gene. Alterations in the expression of α-, β-, γ-catenin, and $p120^{ctn}$ have also been described in a variety of human carcinoma cells (Sommers *et*

*al.*, 1994; Rimm *et al.*, 1995b; Mo and Reynolds, 1996; Hiscox and Jiang, 1997; Morin *et al.*, 1997; Dillon *et al.*, 1998), suggesting a common target in the disruption of E-cad function as a characteristic of cellular invasion.

The aberrant expression of type 1 classical cadherin subtypes may also play a role in the neoplastic transformation of epithelial cells. For example, N-cad expression correlates with the invasive capacity of breast carcinoma and oral squamous carcinoma cell lines whereas relatively non-invasive cell lines derived from these tumourigenic lesions express E-cad (Islam *et al.*, 1996; Hazan *et al.*, 1997). The transfection of a full-length N-cad cDNA into well-differentiated squamous epithelial cells resulted in the disruption of cell-cell interactions, the loss of endogenous E-cad expression, and promoted a scattered fibroblastic phenotype in these cells (Islam *et al.*, 1996; Kim *et al.*, 2000). The down-regulation of endogenous E-cad expression does not appear to be a prerequisite for this morphological transformation, as ectopic N-cad expression in breast carcinoma cells was capable of promoting the invasive capacity of these cells in the absence of any effect on the expression of E-cad (Nieman *et al.*, 1999a; Hazan *et al.*, 2000). Collectively, these data suggest that the differential expression of cadherin subtypes regulates the cellular processes that are involved in tumour cell invasion and may play a role in both promoting and restraining trophoblast invasion during human implantation.

### *1.4.2: Type 2 classical cadherins*

#### *1.4.2-A: Characterization and structure of the type 2 classical cadherins*

The type 2 classical cadherins were first identified in the rat brain using a reverse transcription-polymerase chain reaction (RT-PCR) strategy based on the high degree of homology between two conserved amino acid sequences (TAPPYD and FKKLAD; A=alanine,

D=asparagine, F=phenylalanine, K=lysine, L=leucine, P=proline, T=threonine, and Y=tyrosine) of the classical cadherin cytoplasmic domain (Suzuki *et al.*, 1991). Degenerate oligonucleotides encoding for these two sequences were employed as primers in an RT-PCR reaction using a rat brain cDNA library. This approach led to the identification of eight novel classical cadherin subtypes, termed cad-4 to –11. Detailed analysis of the cDNA clone for cad-4 revealed that this cadherin subtype was the mammalian homologue of chicken R-cad (Inuzuka *et al.*, 1991a; Tanihara *et al.*, 1994a; 1994b). However, the amino acid sequence homology of the remaining cDNA clones suggested that these proteins belonged to a distinct subfamily of cadherins, termed the type 2 classical cadherins (Suzuki, 1996). To date, human cad-6, -7, -8, -9, -10, -11, -12, -14, and –20 have been assigned to the type 2 classical cadherin subfamily (Suzuki *et al.*, 1991; Tanihara et al, 1994a; Shimoyama *et al.*, 1995; 2000; Selig *et al.*, 1997; Shibata *et al.*, 1997; Kools *et al.*, 2000).

The type 2 classical cadherins, like the type 1 classical cadherins, are comprised of five extracellular subdomains, a transmembrane domain, and two cytoplasmic subdomains (Suzuki *et al.*, 1991; Tanihara *et al.*, 1994a). The first four extracellular subdomains (EC1-EC4) of the type 2 classical cadherins contain the conserved $Ca^{2+}$-binding sites and amino acid sequences that are characteristic of the cadherin repeat motifs. In addition, the EC5 subdomain of the type 2 classical cadherins harbours the four conserved cysteine residues present in the type 1 classical cadherins.

Although the type 1 and type 2 classical cadherins share a similar extracellular subdomain structure, the over-all amino acid sequence homology between these two subfamilies is low (<40%; Tanihara *et al.*, 1994a; Nollet *et al.*, 2000; Shimoyama *et al.*, 2000). In particular, the EC1 subdomain, which demonstrates > 65% amino acid homology among members of the type 1 classical cadherins, shows < 50% amino acid homology when compared to the EC1 subdomain of the type 2 classical cadherins. In contrast, the EC1 subdomain of the type 2

classical cadherins exhibits a high degree of amino acid similarity (>75%) among members of this subfamily. Furthermore, the type 2 classical cadherins do not contain the CAR sequence, HAV. The functional significance of these amino acid sequence differences remains to be determined. However, the transfection of full-length cDNAs for several type 2 classical cadherin subtypes has been shown to promote the aggregation of murine fibroblastic L cells, demonstrating that these cadherins are capable of mediating $Ca^{2+}$-dependent cell adhesion in a homophilic manner (Kimura *et al.*, 1995; Sugimoto *et al.*, 1996; Mbalaviele *et al.*, 1998; Shimoyama *et al.*, 1999; 2000).

The transmembrane domain of the type 2 classical cadherins also distinguishes this subfamily from the type 1 classical cadherins (Huber *et al.*, 1999; Shimomaya *et al.*, 2000). For example, the type 1 classical cadherin transmembrane domain is somewhat variable in length, consisting of 25-35 amino acid residues. In contrast, the transmembrane domain of the type 2 classical cadherins is comprised of exactly 33 amino acid residues which are highly similar among members of this subfamily (Shimoyama *et al.*, 2000). However, the type 1 and type 2 classical cadherins share the conserved leucine residues that are believed to regulate the lateral organization of these integral membrane glycoproteins at the cell surface (Huber *et al.*, 1999).

The cytoplasmic domains of the type 1 and type 2 classical cadherins are highly conserved between members of these two subfamilies (Suzuki *et al.*, 1991; Tanihara *et al.*, 1994a; Nollet *et al.*, 2000; Shimoyama *et al.*, 2000). In particular, the amino acid sequences that comprise the β-/γ-catenin and the $p120^{ctn}$ binding regions are present in the cytoplasmic domains of all members of the type 1 and type 2 classical cadherins identified to date. Furthermore, the type 2 classical cadherins have been shown to interact with β-catenin in human breast, gastric, and renal cancer cell lines, primary osteoblast cultures, and MDCK cells (Shibata *et al.*, 1996; 1997; Paul *et al.*, 1997; Cheng *et al.*, 1998; Pishvaian *et al.*, 1999; Stewart *et al.*, 2000). Despite these similarities, the intervening amino acid sequences between the two catenin binding regions

are less well conserved among the members of the type 2 classical cadherins. Collectively, these observations have led to the proposal that the cytoplasmic domains of the type 2 classical cadherins might interact with distinct cytoplasmic proteins and consequently mediate different cellular processes than the type 1 classical cadherins (Nollet *et al.*, 2000).

*1.4.2-B: Cell biology of the type 2 classical cadherins*

The cell biology of the type 2 classical cadherins remains poorly characterized. As the type 2 classical cadherins were first identified in the rodent brain (Suzuki *et al.*, 1991), the majority of studies to date have focused on the role(s) of these cadherin subtypes in the morphogenesis of the mammalian central nervous system (CNS). Several type 2 classical cadherin subtypes, including cad-6, -8, -11, and -12, are differentially expressed in the rodent brain and these expression patterns correlate with the formation of interconnected neuronal cell populations and synaptogenesis (Kimura *et al.*, 1996; Redies and Takeichi, 1996; Inoue *et al.*, 1997; Korematsu and Redies, 1997; Selig *et al.*, 1997; Suzuki *et al.*, 1997). These observations have led to the proposal that the complexity of neuronal circuitry in the mammalian CNS may be mediated, at least in part, by the spatiotemporal expression of the type 2 classical cadherin subtypes in this tissue.

Cad-11 is expressed in several mesenchymal cell subpopulations and in the epithelial cells of the renal tubules during rodent embryonic development (Hoffmann and Balling, 1995; Kimura *et al.*, 1995; Simonneau *et al.*, 1995; Cho *et al.*, 1998). Recently, cad-11 expression has been correlated with the terminal differentiation of rabbit corneal fibroblastic cells and human endometrial stromal cells *in vitro* (Chen *et al.*, 1999; Masur *et al.*, 1999). The overexpression of cad-11 in *Xenopus* embryos was capable of disrupting anterior-posterior axis formation in a manner similar to that observed following the ectopic expression of other type 1 classical

cadherins in these embryos (Dufour *et al.*, 1994; Kuhl *et al.*, 1996; Hadeball *et al.*, 1998). Finally, the transfection of a full-length cad-11 cDNA into murine embryonic fibroblasts increased vascular endothelial growth factor (VEGF) mRNA levels in these cell cultures (Orlandini and Oliviero, in the press). Taken together, these observations suggest that cad-11 is capable of activating intracellular signaling pathways, thereby modulating the differentiation of these cells. To date, the ability of cad-11 to regulate cellular differentiation in humans remains to be determined.

Recent studies have examined the expression of cad-11 during the formation of murine and human bones. For example, cad-11 mRNA transcripts have been detected in bone marrow-derived osteogenic precursor cells as well as in differentiated osteoblasts *in vitro* (Okazaki *et al.*, 1994; Cheng *et al.*, 1998; Lecanda *et al.*, 2000; Shin *et al.*, 2000). The addition of inhibitory peptides containing the HAV sequence only partially blocked the aggregation and differentiation of isolated bone marrow-derived osteoblastic precursor cells, which also express the type 1 classical cadherins N- and R-cad *in vitro* (Cheng *et al.*, 1998; Lecanda *et al.*, 2000). These observations have been taken as indirect evidence of a role for cad-11 in this developmental process. Furthermore, cad-11 expression decreases in the osteoblastic precursor cells of aging rabbits, suggesting that this CAM is capable of maintaining bone tissue integrity in adults (Goomer *et al.*, 1998). However, the functional role(s) of cad-11 in oesteoblast differentiation remains to be elucidated.

The regulated expression of cad-6 in the rodent and human kidney suggests that this CAM plays a key role in the formation and organization of this tissue during development (Xiang *et al.*, 1994; Paul *et al.*, 1997; Cho *et al.*, 1998; Shimazui *et al.*, 2000). In particular, the onset of cad-6 expression correlates with the formation of a polarized epithelium in the kidney (Paul *et al.*, 1997; Cho *et al.*, 1998). Function-perturbing antibodies specific for cad-6 are capable of inhibiting the formation of renal epithelial cell structures *in vitro* (Cho *et al.*, 1998).

Although cad-6 null mutant mice survive until adulthood, these animals exhibit minor defects in the onset of epithelial differentiation in the kidney that lead to an over-all reduction in the number of nephrons in this tissue (Mah *et al.*, 2000). Cad-6 has also been shown to mediate the heterotypic interactions that form between osteoclasts and the surrounding stromal cells *in vitro*, a cellular event that is believed to promote osteoclast differentiation and bone morphogenesis (Mbalaviele *et al.*, 1998). In view of these observations, it is tempting to speculate that this type 2 classical cadherin subtype is as a key morphoregulator during murine embryonic development.

A role for the type 2 classical cadherins has also been suggested in tumorigenesis. For example, cad-6 has been detected in several rodent and human carcinoma cell lines, including the liver, lung, prostate, and kidney (Xiang *et al.*, 1994; Shimoyama *et al.*, 1995; Shimazui *et al.*, 1996; 2000; Paul *et al.*, 1997; Bussemakers *et al.*, 2000). In addition, cad-6 is expressed in the proximal renal tubule epithelium in the normal adult kidney, which is believed to give rise to the majority of renal carcinomas in the human (Holthofer *et al.*, 1983; Paul *et al.*, 1997; Shimazui *et al.*, 2000). These expression patterns have led to the proposal that cad-6 plays a key role in the neoplastic transformation of these cells and may serve as a cellular marker for malignancy in renal-derived tumours (Paul *et al.*, 1997; Shimazui *et al.*, 1998). Cad-11 expression has also been detected in various carcinoma cells, including bone, breast, gastric, prostate, and renal cancer cell lines (Shibata *et al.*, 1996; Shimazui *et al.*, 1996; Kashima *et al.* 1999; Pishvaian *et al.*, 1999; Bussemakers *et al.*, 2000; Tomita *et al.*, 2000). The coordinate expression of cad-11 in these carcinoma cells and the surrounding stroma suggests that this CAM may mediate carcinoma-stromal cell interactions during tumour cell invasion (Shibata *et al.*, 1996; Pishvaian *et al.*, 1999; Tomita *et al.*, 2000). In view of these observations, it is highly likely that the neoplastic transformation of epithelial-derived tumour cells involves a regulated switch in the expression of different classical cadherin subtypes in a manner similar to that observed during tissue morphogenetic processes.

*1.4.3: Unclassified classical cadherins*

Cad-5 (also known as VE-cadherin), cad-15 (also known as M-cadherin), and cad–19 have been included in the classical cadherin gene superfamily but exhibit unique amino acid sequence characteristics, suggesting that these cadherin subtypes are independent members of this gene superfamily of CAMs (Heimark *et al.*, 1990; Donalies *et al.*, 1991; Tanihara *et al.*, 1994a; 1994b; Shimoyama *et al.*, 1998; Kools *et al.*, 2000; Nollet *et al.*, 2000). For example, cad-5 and cad-19 are both type 1 integral membrane glycoproteins that exhibit a similar subdomain structure to the type 2 classical cadherins but lack the conserved amino terminal domain that is post-translationally cleaved during the biosynthesis of these cadherin subtypes (Tanihara *et al.*, 1994b; Kools *et al.*, 2000). In addition, the extracellular and cytoplasmic domains are less well conserved between these two cadherin subtypes and the type 2 classical cadherins.

Cad-5 was originally identified in isolated bovine aortic endothelial cells using a monoclonal antibody that was shown to inhibit $Ca^{2+}$-dependent interactions in these primary cell cultures (Heimark *et al.*, 1990). Further studies revealed that this classical cadherin subtype was expressed exclusively by endothelial cells in the human (Lampugnani *et al.*, 1992). This CAM is capable of interacting with α-, β-, γ-catenin, and $p120^{ctn}$ and mediates junctional complex assembly in isolated human endothelial cells (Lampugnani *et al.*, 1995; Haselton and Heimark, 1997; Navarro *et al.*, 1998). Homozygous null mutant embryos for the cad-5 gene die from severe vascular defects, strongly supporting a role for this CAM in vascular morphogenesis during development (Gory-Faure *et al.*, 1999).

Although the EC1 subdomain of cad-15 demonstrates a relatively high amino acid sequence homology to E-cad (62%), this CAM also lacks the conserved amino terminal pre-

protein domain, the type 1 classical cadherin CAR sequence, and exhibits only 52% amino acid homology between the cytoplasmic domains of these classical cadherin subtypes (Shimoyama *et al.*, 1998). Cad-15 was originally identified using an RT-PCR based approach in murine myoblast cell cultures (Donalies *et al.*, 1991). Cad-15 is capable of mediating $Ca^{2+}$-dependent cell adhesion in a homophilic manner and interacts with α-, β-, and γ-catenin in murine myoblasts *in vitro* (Kuch *et al.*, 1997; Shimoyama *et al.*, 1998). These cad-15/catenin complexes not only link this CAM to the actin-based microfilaments but also interact with the microtubules of these skeletal muscle cells (Kaufmann *et al.*, 1999). Furthermore, the addition of inhibitory peptides specific for cad-15 to cultures of rodent myoblast cells was capable of inhibiting the terminal differentiation and fusion of these cells, suggesting a role for this CAM in the formation of multinucleated myotubes in skeletal muscle (Zeschnigk *et al.*, 1995).

### *1.4.4: Nonclassical cadherins*

#### *1.4.4-A: Truncated cadherins*

Cad-13 (also known as H-cadherin), along with its chicken homologue, T-cadherin, cad-16 (also known as Ksp-cadherin), and cad-17 (also known as HPT-1 cadherin), along with its rat homologue, LI-cadherin, constitute a unique subfamily of cadherins which contain the conserved cadherin repeat motifs in the extracellular domain but lack the entire cytoplasmic domain (Ranscht and Dours-Zimmermann, 1991; Berndorff *et al.*, 1994; Dantzig *et al.*, 1994; Thomson *et al.*, 1995; Lee *et al.*, 1996). Although the EC1 subdomain of cad-13 harbours the type 1 classical cadherin CAR sequence, HAV, this cadherin subtype does not contain a portion of the transmembrane domain and is integrated into the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor (Ranscht and Dours-Zimmermann, 1991). This GPI anchor

results in the targeting of cad-13 to the apical and not the basolateral membrane domain of MDCK cells (Koller and Ranscht, 1996). However, this cadherin subtype is capable of mediating $Ca^{2+}$-dependent cell adhesion in a homophilic manner in transfected Chinese hamster ovary (CHO) cells and is believed to play a role in inhibiting the invasive capacity of breast cancer cells *in vitro* and *in vivo* (Vestal and Ranscht, 1992; Lee *et al.*, 1996; 1998). Cad-16 and -17 not only mediate $Ca^{2+}$-dependent cell adhesion but also interact with a renal $Na^{+}/HCO_3^{-}$ transporter and a liver peptide transporter, respectively (Dantzig *et al.*, 1994; Thomson *et al.*, 1995; 1998; Kreft *et al.*, 1997). Collectively, these observations suggest that the truncated cadherin subtypes play a role(s) in cellular processes that have not reported for other cadherin subtypes.

*1.4.4-B: Desmosomal cadherins*

The desmosomal cadherins have been subdivided into two groups of related glycoproteins; the desmogleins (Dsg) and the desmocollins (Dsc; Buxton *et al.*, 1993). To date, Dsg-1, -2, and –3 and Dsc-1, -2, and –3 have been identified as members of the cadherin gene superfamily in several mammalian species, including humans (Koch *et al.*, 1990; Holton *et al.*, 1990; Arnemann *et al.*, 1991; 1992a; 1992b; Collins *et al.*, 1991; Mechanic *et al.*, 1991; Nilles *et al.*, 1991; Amagai *et al.*, 1991; King *et al.*, 1993). In addition, differential pre-mRNA splicing gives rise to two distinct isoforms of the Dsc glycoproteins, a phenomenon that is not observed for the type 1 classical cadherins (Collins *et al.*, 1991; Parker *et al.*, 1991; Thies *et al.*, 1993). Like the classical cadherins, the desmosomal cadherins are type 1 integral membrane glycoproteins, exhibit partial amino acid homology among their extracellular domains (>50%), and contain the conserved $Ca^{2+}$-binding domains that are present at the interface between the cadherin repeat motifs. However, the cytoplasmic domains are highly divergent between the desmosomal cadherins and the classical cadherins and also among the Dsg and Dsc subtypes

(Collins *et al.*, 1991; Wheeler *et al.*, 1991; Koch *et al.*, 1991). In particular, all three Dsg subtypes contain longer cytoplasmic domains than Dsc and exhibit amino acid similarity in only a specific region that has been shown to interact with γ-catenin, but not β-catenin (Kowalczyk *et al.*, 1996; Witcher *et al.*, 1996; Wahl *et al.*, 2000).

Although the Dsg and Dsc isoforms are capable of forming complexes with γ-catenin, these desmosomal cadherins interact with the intermediate filaments and not the actin-based microfilaments of the cytoskeleton (Cowin *et al.*, 1986; Franke *et al.*, 1989; Troyanovsky *et al.*, 1994). The desmosomal cadherin binding region of γ-catenin is distinct from that of the classical cadherins and is adjacent to the α-catenin binding region, likely inhibiting the ability of this cytoplasmic protein to interact with α-catenin and the microfilaments of the cytoskeleton when it is complexed to the different Dsg and Dsc subtypes (Ozawa *et al.*, 1995; Sacco *et al.*, 1995; Chitaev *et al.*, 1996; Kowalczyk *et al.*, 1996; Troyanovsky *et al.*, 1996; Wahl *et al.*, 1996; Witcher *et al.*, 1996). Instead, desmoplakin, which is capable of interacting with intermediate filaments, is localized to desmosomal junctions in cultured epithelial cells (Stappenbeck and Green, 1992; Kowalczyk *et al.*, 1994; Schmidt *et al.*, 1994). As γ-catenin is capable of binding directly to both desmoplakin and the cytoplasmic domains of the desmosomal cadherins, this catenin subtype likely serves as an intermediate protein in linking this cadherin/catenin complex to the cytoskeleton (Kowalczyk *et al.*, 1997).

Desmosome function has been best characterized in the murine and human epidermis. For example, autoantibodies directed against Dsg3 have been shown to result in the desmosomal plaque disruption that is observed in the skin blistering disease, *pemphigus vulgaris* (Amagai *et al.*, 1991; Silos *et al.*, 1996). Furthermore, targeted disruption of the Dsg3 gene in mice resulted in the development of skin defects similar to those observed in this disease of the epidermis (Koch *et al.*, 1997). Despite the proposed role for the Dsg and Dsc subtypes in mediating

adhesive interactions, functional studies have failed to clearly support this hypothesis. For example, some studies have reported that the transfection of full-length Dsg1 and Dsc2 into murine fibroblastic L cells failed to confer significant aggregation in these cultures (Chidgey *et al.*, 1996; Kowalczyk *et al.*, 1996). In contrast, the co-transfection of these desmosomal cadherin subtypes with γ-catenin resulted in the formation of strong intercellular contacts in these same cells, suggesting that this catenin subtype is a key regulator of desmosomal-mediated adhesion (Marcozzi *et al.*, 1998).

*1.4.4-C: Protocadherins*

The first two members of the protocadherin (Pcdh) subfamily were identified using an RT-PCR based screen of rat and human brain cDNA libraries and were subsequently named Pcdh1 and Pcdh2 (Sano *et al.*, 1993). This subfamily of cadherins is characterized by the presence of more than four cadherin repeat motifs in their extracellular domain, the absence of the four conserved cysteine residues in the membrane proximal extracellular subdomain, and a cytoplasmic domain that is highly divergent from that of the classical cadherins (Suzuki, 2000). Pcdh1 and Pcdh2 have been shown to mediate $Ca^{2+}$-dependent cellular interactions in a homophilic manner, although these interactions are weaker than those observed for the classical cadherins and are independent of interactions with the catenins (Sano *et al.*, 1993).

More recent studies have determined that as many as 60 proteins belong to the protocadherin subfamily and are likely to be further subdivided into several subfamilies based on their unique structural characteristics (Nollet *et al.*, 2000). For example, a novel subfamily of protocadherins was identified in the mouse brain based on their ability to interact with the *src* tyrosine kinase, Fyn, a protein implicated in the long term potentiation of synapses and memory function in rodents (Grant *et al.*, 1992; Kohmura *et al.*, 1998). These protocadherin subtypes,

designated cadherin-related neuronal receptors (CNR), have been localized to synaptic junctions and are believed to play a role in modulating interneuronal connections and signal transduction in the CNS. Wu and Maniatis (1999) have since identified 52 novel cadherin-related genes based on a homology screen of genomic sequences submitted to the GenBank. These protocadherins are organized into three closely linked gene clusters on human chromosome 5 and have consequently been divided into three subgroups; Pcdhα, Pcdhβ, and Pcdhγ. Finally, a protocadherin-related molecule, designated Flamingo, has been identified in *Drosophila* (Iwai *et al.*, 1997; Usui *et al.*, 1999), the mouse (Hadjantonakis *et al.*, 1998), and the rat (Nakayama *et al.*, 1998) and is predicted to contain seven transmembrane domains. Although the biological significance of this marked structural diversity has yet to be determined, these observations strongly suggest multiple roles for these distinct members of the cadherin gene superfamily during development and in adults.

*1.4.4-D: Unclassified cadherin-related proteins*

Several studies have determined that the *Drosophila* and human FAT proteins contain cadherin repeat motifs in their extracellular domains (Mahoney *et al.*, 1991; Clark *et al.*, 1995; Dunne *et al.*, 1995). However, these type 1 integral membrane glycoproteins contain 34 tandemly arranged cadherin repeat motifs and exhibit low amino acid homology (<35%) with other classical cadherins. FAT is believed to be the product of a tumour suppressor gene as a recessive mutation in this gene leads to the hyperplastic growth of cells in the wing bud of *Drosophila* embryos (Bryant *et al.*, 1988; Mahoney *et al.*, 1991). FAT is also expressed by epithelial cells, fibroblasts, and smooth muscle cells in the human but the role(s) of this protein in these cells remains to be determined (Dunne *et al.*, 1995; Matsuyoshi and Imamura, 1997). More recent studies have identified a novel cadherin subtype, termed CDH23 (also known as

otocadherin), as a gene that is frequently mutated in human Usher syndrome type 1D, an autosomal recessive disorder that is characterized by profound auditory and visual impairment (Keats and Corey, 1999; Bolz *et al.*, 2001). CDH23 is a type 1 integral membrane glycoprotein that contains 27 cadherin repeat motifs and has been shown to regulate ciliary function in neuroepithelial hair cells of the murine ear (Bolz *et al.*, 2001; Palma *et al.*, 2001).

The transmembrane receptor tyrosine kinase, RET, also contains cadherin repeat motifs in its extracellular domain (Takahashi and Cooper, 1987; Schneider, 1992; Iwamoto *et al.*, 1993). However, RET has not been shown to mediate cell adhesion and exhibits low amino acid similarity (< 30%) with other cadherin subtypes, suggesting that this receptor is only distantly related to the cadherin gene superfamily (Takahashi *et al.*, 1993). In view of the central role that RET plays in the neoplastic transformation of carcinoma cells (Pasini *et al.*, 1996), it is tempting to speculate that this proto-oncogene is capable of interacting with and/or regulating the function of the other cadherin subtypes at the cell surface.

### 1.5: Identification of the classical cadherins present in the human placenta and during trophoblast differentiation

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 in adults, it would seem likely that members of this gene superfamily mediate human implantation and placentation.

The expression of the classical cadherins in the human placenta has been previously described. The type 1 cadherins, E-cad and N-cad, the type 2 cadherin subtype, cad-11, and the unclassified cadherin subtype, cad-5, have been immunolocalized to discrete cell subpopulations in sections of placental tissues obtained from different stages of gestation (Fisher *et al.*, 1989;

Leach *et al.*, 1993; 2000; Babawale *et al.*, 1996; MacCalman *et al.*, 1996a; 1997; Zhou *et al.*, 1997a; Floridon *et al.* 2000). For example, N-cad and cad-5 are expressed by the endothelial cells in the chorionic villi of the human first trimester and term placenta (Leach *et al.*, 1993; 2000; Zhou *et al.*, 1997a). In contrast, E-cad and cad-11 are differentially expressed in the trophoblastic cells of these placental tissues (MacCalman *et al.*, 1996a). In particular, E-cad expression has been detected in the villous cytotrophoblasts, but not the syncytial trophoblast of the human first trimester and term placenta (Fisher *et al.*, 1989; Babawale *et al.*, 1996; Zhou *et al.*, 1997a; Floridon *et al.*, 2000). Our laboratory has recently determined that the type 2 classical cadherin subtype, cad-11, is expressed by the multinucleated syncytial trophoblast layer of these placental tissues (MacCalman *et al.*, 1996a; 1997a).

The expression of the classical cadherins in human extravillous cytotrophoblasts remains contentious. Most studies have described a reduction in the expression of E-cad in the extravillous cytotrophoblast column and invasive extravillous cytotrophoblasts of the first trimester human placenta (Fisher *et al.*, 1989; Babawale *et al.*, 1996; Zhou *et al.*, 1997a), although a more recent study by Floridon *et al.* (2000) has not confirmed these findings. Studies from our laboratory have determined that cad-11 is expressed in the extravillous cytotrophoblast columns, but not the villous cytotrophoblasts at the proximal ends of these cellular columns in the first trimester of pregnancy (MacCalman *et al.*, 1996a; 1997a). Although cad-5 expression is believed to be restricted to endothelial cells (Lampugnani *et al.*, 1992), this CAM has also been detected in the epithelial-derived extravillous cytotrophoblasts of the human first trimester placenta (Zhou *et al.*, 1997a).

Our laboratory has recently determined that E-cad and cad-11 are also differentially expressed during the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta (MacCalman *et al.*, 1996a). In particular, E-cad mRNA levels and protein expression are high in freshly isolated cytotrophoblasts and decrease as these cells

undergo aggregation and fusion to form multinucleated syncytium with time in culture (Coutifaris *et al.*, 1991; Rebut-Bonneton *et al.*, 1993; MacCalman *et al.*, 1996a). The loss of E-cad expression is concomitant with an increase in the levels of the mRNA transcript encoding cad-11 in these primary cultures (MacCalman *et al.*, 1996a). A switch between E-cad and cad-11 expression was also observed in BeWo choriocarcinoma cells undergoing terminal differentiation and fusion in response to the intracellular secondary messenger, cAMP. In contrast, E-cad but not cad-11 was readily detectable in non-fusing JEG-3 cells. Taken together, these observations indicate that cad-11 expression is tightly regulated during the formation of multinucleated syncytium from mononucleate trophoblastic cells *in vitro*.

We have recently extended these observations to the transforming growth factor-β1-mediated aggregation and fusion of extravillous cytotrophoblasts propagated from human first trimester placental explants (Appendix I). In our initial studies, mRNA transcripts that encode for cad-11 but not E-cad were detected in these isolated human extravillous cytotrophoblasts (MacCalman *et al.*, 1998). However, we failed to detect significant cad-11 immunoreactivity in these primary cell cultures, using a mouse monoclonal antibody specific for this CAM. Northern and Western blot analysis demonstrated that TGF-β1 was capable of increasing cad-11 mRNA levels and protein expression in isolated extravillous cytotrophoblasts in a dose-dependent manner (Appendix I). The increase in cad-11 expression correlated with the formation of large cellular aggregates and multinucleated cells in these primary cell cultures. Cad-11 expression was subsequently immunolocalized to both of these cellular structures following treatment with TGF-β1.

## 1.6: Hypothesis and rationale

A switch in the expression of the classical cadherin subtypes, from E-cad to cad-11, is associated with the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro* and *in vivo*. As the function of the classical cadherins is regulated, at least in part, by their interactions with the catenins, we hypothesize that the expression of these cytoplasmic proteins is tightly regulated during this developmental process. To date, the identity and expression pattern(s) of the catenin subtypes in the trophoblastic cells of the human placenta remain to be determined. To address these outstanding issues, we examined the expression of α-, β-, γ-catenin, and $p120^{ctn}$ expression during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro* and *in vivo*. These studies not only give us insight into the adhesive mechanisms operative during the formation and organization of the human placenta but also add to our understanding of the role(s) of distinct cadherin/catenin complexes during cellular differentiation.

Cad-11 expression has been correlated with trophoblast differentiation and fusion in three separate *in vitro* model systems: 1) the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta, 2) the cAMP-mediated differentiation and fusion of BeWo choriocarcinoma cells, and 3) the TGF-β1-mediated aggregation and fusion of extravillous cytotrophoblasts propagated from first trimester placental explants. In contrast, mononucleate JEG-3 cells express E-cad but not cad-11. In view of these observations, we hypothesize that cad-11 plays a key role in human placentation by mediating the formation of multinucleated syncytium from mononucleate trophoblastic cells. To address this issue, we determined the ability of ectopic cad-11 expression to mediate the terminal differentiation and fusion of mononucleate JEG-3 cells. In addition, we examined the effects of reduced cad-11 expression on the formation of multinucleated syncytium in primary cultures of villous

cytotrophoblasts isolated from the human term placenta. Collectively, these studies better define the role of cad-11 during this developmental process and provide insight into the cell biology of this type 2 classical cadherin.

The results of these studies are presented in the following sections.

# CHAPTER II: REGULATED EXPRESSION OF α-, β-, γ-CATENIN AND p120$^{ctn}$ DURING THE TERMINAL DIFFERENTIATION AND FUSION OF HUMAN VILLOUS CYTOTROPHOBLASTS

## 2.1: Preface

This chapter describes a series of studies undertaken to characterize the expression patterns of the cadherin-associated proteins, known as the catenins, during syncytial trophoblast formation in the human placenta.

Previous studies have examined E-cad and β-catenin expression during the differentiation of the polarized trophectoderm in murine and bovine blastocysts (Ohsugi *et al.*, 1996; Barcroft *et al.*, 1998). These two cellular proteins have been co-localized in this tissue layer during pre-implantation embryonic development. However, the expression pattern of β-catenin during the formation of the human placenta has not been characterized. In these studies, we examined the expression of β-catenin in primary cultures of human villous cytotrophoblasts undergoing terminal differentiation and fusion *in vitro*. We demonstrated that β-catenin mRNA levels and protein expression were high in freshly isolated villous cytotrophoblasts but declined as these cells underwent terminal differentiation and fusion with time in culture. β-catenin was present in immunoprecipitated complexes containing E-cad, but not cad-11, in these primary cell cultures. In addition, there was no significant difference in the levels of β-catenin expression in non-fusing JEG-3 choriocarcinoma cell cultures at any of the time points examined in these studies. β-catenin was subsequently immunolocalized to the mononucleate cells present in these two trophoblastic cell cultures and the villous cytotrophoblasts of human placental tissues. Collectively, these observations suggest that E-cad/β-catenin complexes regulate villous cytotrophoblast function during this developmental process.

These studies are the first to describe the regulated expression of β-catenin during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro* and *in vivo*. In addition, this is the first demonstration of β-catenin mRNA levels and protein expression being co-ordinately regulated during cellular differentiation. The results of these studies are published in a manuscript entitled "Regulation of β-catenin mRNA and protein levels in human villous cytotrophoblasts undergoing aggregation and fusion *in vitro*: correlation with E-cadherin expression" published in the *Journal of Reproduction and Fertility* (S Getsios, GTC Chen, and CD MacCalman (2000); 119:59-68).



The formation and localization of classical cadherin complexes containing either β- or γ-catenin are differentially regulated during cellular differentiation and development (Butz and Larue, 1995; Lampugnani *et al.*, 1995). As β-catenin expression correlated with the levels of E-cad, but not cad-11 expression during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro*, we examined the expression of the other catenin subtypes, namely α-, γ-catenin, and $p120^{ctn}$, during this developmental process. We determined that α-, γ-catenin, and $p120^{ctn}$ expression were down-regulated during the formation of multinucleated syncytium in a manner similar to β-catenin expression in these primary cell cultures. These four catenin subtypes were immunolocalized to the intercellular borders between villous cytotrophoblasts and the syncytial trophoblast but not the outer syncytial trophoblast surface *in vitro* and *in vivo*.

These studies are the first to characterize the expression of α-, γ-catenin, and $p120^{ctn}$ during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro* and *in vivo*. We believe that this is the first demonstration of the coordinate down-regulation of catenin expression during a cellular differentiation process. The results of these studies are presented in a manuscript entitled "α-, β-, γ-catenin and $p120^{ctn}$ expression during the terminal differentiation

and fusion of human mononucleate cytotrophoblasts *in vitro*" published in *Molecular Reproduction and Development* (S Getsios, GTC Chen, and CD MacCalman; in the press). 

GTC Chen prepared the cell lysates for the Western blot analyses in these studies. The remainder of the research was conducted by S Getsios under the supervision of Dr. CD MacCalman.

## 2.2: β-CATENIN mRNA AND PROTEIN LEVELS ARE REGULATED IN HUMAN VILLOUS CYTOTROPHOBLASTS UNDERGOING AGGREGATION AND FUSION *IN VITRO*: CORRELATION WITH E-CADHERIN EXPRESSION


### Abstract

The cellular mechanisms underlying the formation and organization of the human placenta remain poorly understood. Recent studies have demonstrated that E-cad, in association with the cytoplasmic protein known as β-catenin, plays an integral role in the differentiation of the trophectoderm in the murine and bovine embryo. Although E-cad expression is regulated during the aggregation and fusion of human villous cytotrophoblasts, the expression of β-catenin during the terminal differentiation of these primary cell cultures has not been determined. In this study, β-catenin mRNA concentrations and protein expression were examined in primary cultures of human villous cytotrophoblasts using Northern and Western blot analysis. β-catenin mRNA concentrations and protein expression were high in freshly isolated mononucleate cytotrophoblasts but decreased as these cells underwent aggregation and fusion to form syncytium. A similar pattern of expression was observed for the E-cad mRNA transcript and protein species present in these cell cultures. Immunoprecipitation studies demonstrated that the β-catenin and E-cad protein species present in the mononucleate cytotrophoblasts were capable of forming intracellular complexes. In contrast, β-catenin and E-cad mRNA and protein expression in JEG-3 choriocarcinoma cells remained constant over time in culture. β-catenin and E-cad expression was subsequently immunolocalized to the aggregates of mononucleate cells present in both of these trophoblastic cell cultures and the villous cytotrophoblasts of the human first trimester and term placenta. Taken together, these observations indicate that E-cad/β-catenin complexes play a central role in the terminal differentiation of human trophoblasts *in vitro* and *in vivo*.

## Introduction

The placenta plays a key role in supporting fetal growth (Aplin, 1991). This transient organ is the site of transfer of respiratory gases, nutrients, and waste products between the fetal and maternal systems; it serves as a barrier against blood-borne pathogens and the maternal immune system; and it fulfills an endocrine role by secreting hormones, growth factors and other bioactive substances required for the establishment and maintenance of pregnancy. The majority of these placental functions are performed by the syncytial trophoblast, the outer multinucleated cellular layer of chorionic villi, which is formed by the terminal differentiation and fusion of the underlying mononucleate cytotrophoblasts (Richart, 1961; Kliman *et al.*, 1986). To date, the molecular and cellular mechanisms underlying these developmental processes remain poorly characterized.

Villous cytotrophoblasts isolated from the human term placenta mimic many of the cellular events associated with the formation and organization of the human placenta *in vivo* (Kliman *et al.*, 1986). For example, freshly isolated mononucleate cytotrophoblasts aggregate and establish extensive interactions with one another through the formation of desmosomes, adherens, and gap junctions (Babalola *et al.*, 1990; Douglas and King, 1990; Cronier *et al.*, 1994). The resultant cellular aggregates promote the terminal differentiation and fusion of the mononucleate cytotrophoblasts into multinucleated syncytium. These morphogenetic events are believed to be mediated, at least in part, by the regulated expression of members of the cadherin gene superfamily of $Ca^{2+}$-dependent CAMs in these primary cell cultures.

Two cadherin subtypes, E-cad and cad-11, are differentially expressed during the terminal differentiation of human cytotrophoblasts *in vitro* (MacCalman *et al.*, 1996a). In particular, E-cad mRNA concentrations and protein expression are high in freshly isolated villous cytotrophoblasts and decrease as aggregates are formed and the cells begin to undergo

differentiation and fusion to form multinucleated syncytium (Coutifaris *et al.*, 1991; Rebut-Bonneton *et al.*, 1993). The loss of E-cad expression in these cell cultures is concomitant with an increase in the expression of cad-11 (MacCalman *et al.*, 1996a). Furthermore, function-perturbing antibodies generated against E-cad inhibit aggregation (Coutifaris *et al.*, 1991) whereas antisense oligonucleotides specific for human cad-11 inhibit fusion in these primary cell cultures (MacCalman, 1997a; Chapter 3.2). Taken together, these observations have led to the proposal that these two cadherin subtypes play discrete roles in the aggregation, differentiation, and fusion of villous cytotrophoblasts *in vitro*.

In general, the ability of cadherins to mediate cell-cell interactions is dependent on these CAMs interacting with at least three cytoskeletal-associated proteins known as α-, β-, and γ-catenin (Ozawa *et al.*, 1989; Knudsen and Wheelock, 1992; Kemler, 1993). The cytoplasmic domain of the cadherin interacts with either β- or γ-catenin in a mutually exclusive manner (Butz and Kemler, 1994; Nathke *et al.*, 1994). These two catenins, in turn, bind to α-catenin which is responsible for anchoring the cadherins to the actin filaments of the cytoskeleton either directly (Ozawa *et al.*, 1990) or indirectly through interactions with the actin-binding protein, α-actinin (Knudsen *et al.*, 1995). The catenins not only link the cadherins to the underlying cytoskeleton but are thought to be involved in activating several intracellular signaling pathways (Gumbiner, 1995; Dale, 1998).

Although β-catenin has been colocalized with E-cad in the inner cell mass and trophectoderm of the murine (Ohsugi *et al.*, 1996) and bovine embryo (Barcroft *et al.*, 1998), the expression pattern of this catenin subtype during the formation and organization of the human placenta has not been characterized. The objective of this study was to provide insight into mechanisms by which cadherins mediate the terminal differentiation of human trophoblasts. β-catenin and E-cad mRNA concentrations and protein expression were examined in primary

cultures of villous cytotrophoblasts undergoing aggregation, differentiation, and fusion *in vitro* using Northern and Western blot analysis, respectively. Similarly, β-catenin and E-cad mRNA concentrations and protein expression were examined in cultures of JEG-3 cells, a choriocarcinoma cell line which does not undergo terminal differentiation and fusion under standard culture conditions. β-catenin and E-cad expression was subsequently localized in these trophoblastic cell cultures, as well as in the first trimester and term placenta *in vivo*, using immunocytochemistry. Immunoprecipitation studies were performed using cell lysates prepared from primary cultures of villous cytotrophoblasts isolated from the human term placenta to determine whether β-catenin and E-cad were capable of forming intracellular complexes in human trophoblasts.

## Materials and Methods

### *Tissues*

First trimester (approximately 12 weeks of gestation; n=3) and term placental tissues (n=6) were obtained from women undergoing elective termination of pregnancy and cesarean section, respectively. Research on these human placental tissues was approved by the Committee for Ethical Review of Research Involving Human Subjects, University of British Columbia, Vancouver, Canada and was performed with the informed consent of the patients.

### *Cell preparation and culture*

Villous cytotrophoblasts were prepared from human term placentae as previously described by Kliman *et al.* (1986). This method, which utilizes serial trypsin-DNase digestions

in $Ca^{2+}$ and $Mg^{2+}$-free media followed by Percoll gradient centrifugation to purify cells, yields a highly enriched preparation of cytotrophoblasts.

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) were harvested from ongoing cultures with 0.125 % trypsin in EDTA buffer.

Villous cytotrophoblasts and JEG-3 cells were cultured in 100 mm culture dishes at densities of $1.2 \times 10^7$ cells per dish or $5 \times 10^6$ cells per dish, respectively. For immunocytochemistry, the cells were seeded on glass coverslips (2 x 2 cm) that had been placed in 35 mm culture dishes at densities of $5 \times 10^5$ cells per dish or $2 \times 10^5$ cells per dish for the villous cytotrophoblasts and JEG-3 cells, respectively. Culture media (Dulbecco's modified Eagle's medium; DMEM (Gibco BRL, Burlington ON) containing 25 mM glucose, 25 mM HEPES and 50 μg/ml gentamicin) was supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone Labs Inc., Logan, UT).

*Northern Blot Analysis*

Total RNA was prepared from villous cytotrophoblasts or JEG-3 cells that had been cultured for 0, 12, 24, 48 or 72 h using the phenol-chloroform method of Chomczynski and Sacchi (1987). The RNA species were resolved by electrophoresis in 1% agarose gels containing 3.7% formaldehyde. Approximately 20 μg of total RNA was loaded in each lane. The fractionated RNA species were then transferred onto charged nylon membranes.

The Northern blots were probed with radiolabeled cDNAs specific for human β-catenin (a gift from S. Byers, Georgetown University, Washington, DC), E-cad, and a synthetic oligonucleotide specific for 18S rRNA according to the methods of Getsios *et al.* (1998b; Appendix I).

*Detergent Extraction, Western Blot Analysis, and Immunoprecipitation*

Cultures of villous cytotrophoblasts and JEG-3 cells were washed three times in PBS and incubated in 1 ml cell lysis buffer (10 mM Tris HCl, pH 7.5 containing 0.5% Nonidet P-40 (NP-40), 0.5 mM $CaCl_2$ and 1.0 mM phenylmethylsulfonyl fluoride (PMSF)) at 4 $^{o}$C for 30 min on a rocking platform. The cell lysates were centrifuged at 10,000 x g for 20 min and the supernatants used for immunoprecipitation or Western blot analysis. The concentration of protein in the cell lysates was determined using the BCA kit (Pierce Chemicals, Rockford, IL).

Western blots containing aliquots (20 μg) of the cell lysates extracted from villous cytotrophoblasts or JEG-3 cells that had been cultured for 12, 24, 48, or 72 h were prepared according to the methods described by MacCalman *et al.* (1993). The nitrocellulose blots were probed with a mouse monoclonal antibody directed against human E-cad or β-catenin (Transduction Labs, Lexington, KY).

Immunoprecipitations were performed according to a protocol modified from Pishviain *et al.* (1999). Briefly, aliquots (250 μg) of cell lysates, prepared from villous cytotrophoblasts that had been cultured for 48 h, were pre-cleared with 10 μl of a protein A-agarose conjugate (2 mg/ml; Transduction Labs) for 30 min. The agarose was removed from the cell lysates by centrifugation at 10,000 x g for 4 min. The resultant supernatants were incubated with 5 μg of a mouse monoclonal antibody directed against either human E-cad, β-catenin (Transduction Labs), or cad-11 (C11-113H, ICOS Corp., Bothell, WA) for 1 h. The immune complexes were incubated with 5 μg of a rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 30 min. Protein A-agarose was added to the lysates and the reaction mixture incubated for an additional 30 min. The agarose-bound immune complexes were centrifuged at 10,000 x g for 4 min, washed five times with cell lysis buffer, resuspended in Laemmli sample

lysis buffer, and resolved by SDS-PAGE under reducing condition. The proteins were transferred electrophoretically from the gels onto nitrocellulose paper. The immunoblots were probed with a mouse monoclonal antibody directed against human β-catenin (Transduction Labs). The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen.

*Immunolocalization of E-cad and β-catenin in trophoblastic cell cultures and human placental tissues.*

Frozen sections (8 μm) prepared from human first trimester and term placental tissues and cultures of villous cytotrophoblasts or JEG-3 cells grown on glass coverslips for 12 or 72 h, were fixed in 4% paraformaldehyde for 15 min at room temperature (RT).

The fixed tissue sections and cells were immunostained using mouse monoclonal antibodies directed against either human E-cad or β-catenin (Transduction Labs) according to the methods described by Getsios *et al.* (1998b; Appendix I).

*Statistical Analysis*

The results are presented as the mean relative absorbance (± SEM) for three independent experiments. Statistical differences between time points were assessed by the analysis of variance (ANOVA). Differences were considered significant for $P$ values $\leq 0.05$. Significant differences between the means were determined using the least significant difference test.

**Results**

A single E-cad mRNA transcript of 5.6 kb was detected in all of the total RNA extracts prepared from the primary cultures of villous cytotrophoblasts (Fig. 2.2.1). The concentration of this E-cad mRNA transcript was high in freshly isolated cytotrophoblasts. However, as reported in other studies (Coutifaris *et al.*, 1991; MacCalman *et al.*, 1996a), there was a significant decrease in E-cad mRNA concentration between 24 and 48 h of culture, the period of time in which these cells begin to undergo terminal differentiation and fusion *in vitro*. There was a further decrease in E-cad mRNA concentration at 72 h of culture as the cells continued to form multinucleated syncytium. Similarly, maximum levels of the β-catenin mRNA transcript (3.3 kb) were detected in freshly isolated cytotrophoblasts and there was a significant decrease in the concentration of this mRNA transcript at 48 h. β-catenin mRNA concentration continued to decrease until the end of the experiment at 72 h.

Western blot analysis using mouse monoclonal antibodies directed against either human E-cad or β-catenin and extracts prepared from cultured villous cytotrophoblasts revealed a single E-cad (120 kDa) and β-catenin (92 kDa) protein species in all of the cellular extracts (Fig. 2.2.2). In agreement with the Northern blot analysis, there was a significant decrease in the expression of these two protein species at 48 h of culture. There was a further decrease in E-cad and β-catenin expression as the cytotrophoblasts continued to undergo differentiation and fusion to form syncytium over time in culture.

The E-cad and β-catenin mRNA transcripts and protein species were also readily detectable in extracts prepared from JEG-3 cells (Figs. 2.2.3 and 4, respectively). However, in contrast to the primary cultures of villous cytotrophoblasts, there were no significant differences in E-cad or β-catenin mRNA concentrations and protein expression in the JEG-3 cell cultures at any of the time points examined.

Intense immunostaining for E-cad and β-catenin expression was detected in mononucleate cytotrophoblasts. In particular, E-cad and β-catenin were localized to areas of cell-cell contact in the mononucleate cellular aggregates that formed in these primary cell cultures (Fig. 2.2.5). After fusion, E-cad and β-catenin immunostaining was not detectable on the surface of the multinucleated syncytium with the exception of areas that were in direct contact with unfused mononuclear cytotrophoblasts. In contrast, E-cad and β-catenin immunostaining was readily detectable in areas of contact between JEG-3 cells cultured for 72 h.

Immunoprecipitation studies using cell lysates prepared from villous cytotrophoblasts cultured for 48 h demonstrated that E-cad and β-catenin were capable of forming intracellular complexes in these primary cell cultures (Fig. 2.2.6). In contrast, β-catenin was not detected in cell lysates that had been immunoprecipitated with a mouse monoclonal antibody directed against cad-11. The presence of a 125 kDa cad-11 protein species, previously detected in primary cultures of villous cytotrophoblasts (MacCalman *et al.*, 1996a), was confirmed by immunoblotting.

E-cad and β-catenin were also localized to the mononucleate villous cytotrophoblasts present in the human first trimester and term placenta (Fig 2.2.7). In contrast, significant expression of either E-cad or β-catenin was not detected in the outer syncytial trophoblast layer of these placental tissues. However, ribbon-like fragments of immunodetectable E-cad and β-catenin were occasionally observed in the inner surface of the syncytial trophoblast of the term placenta, primarily in regions adjacent to the mononucleate cytotrophoblasts present in this tissue. This immunostaining pattern has been previously reported for E-cad expression in BeWo cells undergoing fusion in response to the secondary intracellular messenger, cAMP (Coutifaris *et al.*, 1991).

## Discussion

A single β-catenin mRNA transcript of 3.3 kb was detected in all of the total RNA extracts prepared from the villous cytotrophoblast and JEG-3 choricarcinoma cell cultures. This β-catenin mRNA transcript has been previously detected in human endometrial stromal cells (Chen *et al.*, 1998a) and breast, gastric, and colon carcinoma cell lines (Oyama *et al.*, 1994; Munemitsu *et al.*, 1995; Byers *et al.*, 1996). Similarly, a 92 kDa protein species corresponding to β-catenin has been detected in a wide variety of tissues and cells, including *Xenopus* embryos, human liver, epidermoid cells, and murine neuroblastoma cell lines (McCrea and Gumbiner, 1991; Aberle *et al.*, 1997; Miyoshi *et al.*, 1998). However, the present study is the first to demonstrate that β-catenin mRNA concentration and protein expression are coordinately regulated during the terminal differentiation of a mammalian cell *in vitro*.

Cadherin-mediated cellular interactions govern the developmental fate of cells and the subsequent formation of tissues (Larue *et al.*, 1996; Redfield *et al.*, 1997; Linask *et al.*, 1998). These developmental processes may be mediated, at least in part, by intracellular signaling via the cadherin/catenin complex (Gumbiner, 1995; Dale, 1998). The results of the present study demonstrate that E-cad and β-catenin mRNA concentration and protein expression are high in freshly isolated villous cytotrophoblasts and decrease as these cells undergo aggregation and fusion to form syncytium *in vitro*. In contrast, E-cad and β-catenin expression levels were maintained in non-fusing JEG-3 cultures. These findings indicate that the loss of E-cad and β-catenin expression in human trophoblastic cells may alter many characteristics of these cells, including the integrity and organization of intercellular junctional complexes (Babalola *et al.*, 1990; Douglas and King, 1990; Cronier *et al.*, 1994), remodeling of the cytoskeleton (Douglas and King, 1993) and the expression of transcription factors (Dakour *et al.*, 1999), all of which,

alone or in combination, are likely to impact on cellular and tissue differentiation *in vitro* and *in vivo*. At present, the molecular and cellular mechanisms involved in coordinately regulating cadherins and catenins in a mammalian cell remain poorly characterized. Although several studies suggest that cadherins modulate catenin expression, these observations have been based primarily on the examination of catenin concentration after the transfection of exogenous cadherins (Redfield *et al.*, 1997; Sadot *et al.*, 1998).

E-cad and β-catenin expression was localized to villous mononucleate cytotrophoblasts *in vitro* and *in vivo*. In addition, the present study indicates that E-cad and β-catenin form functional complexes in these human placental cells. This cadherin/catenin complex appears to play a central role in the formation and organization of the murine placenta. In particular, E-cad null mutant mouse embryos die at the time of implantation due to their inability to form a functional trophectoderm (Larue *et al.*, 1994). In contrast, trophectoderm formation does not appear to be effected in β-catenin null mutant mice before day 7.5 of pregnancy (Haegel *et al.*, 1995). However, the early stages of embryonic development in these null mutant mouse strains are thought to result from the persistence of maternally derived gene products. The ability of β-catenin null mutant mice to form a trophectoderm may also be attributed to the increase in γ-catenin expression levels observed in this cell layer. Furthermore, instead of being restricted to the desmosomes, which form in this cellular layer during embryonic development (Fleming *et al.*, 1991), γ-catenin was colocalized with E-cad to the basolateral membrane domain of the epithelial cells forming the trophectoderm of these null mutant mice (Ohsugi *et al.*, 1997). Taken together, these observations indicate that γ-catenin can be substituted, at least in part, for β-catenin, thereby promoting E-cad-mediated cellular interactions during the development of the trophectoderm in these mouse embryos. Although these placental structures appear to be morphologically normal, their ability to sustain a pregnancy and the growth and development of

the fetus after day 7.5 of pregnancy has not been determined, primarily due to the morphological defects observed in the inner cell mass of β-catenin null mutant embryos.

β-catenin may also mediate cellular differentiation and mammalian development through intracellular signal transduction pathways that are independent of the cadherins. For example, alterations in β-catenin expression have been associated with the formation of mesoderm in the *Xenopus* and murine embryo (Heasman *et al.*, 1994; Haegel *et al.*, 1995). In addition, intracellular signaling by wnt growth factors results in an accumulation of β-catenin in the cytoplasm and nucleus of *Drosophila* embryonic cells and MDCK cells (Peifer *et al.*, 1994b; Behrens *et al.*, 1996). Although the role of β-catenin in the terminal differentiation and fusion of villous cytotrophoblasts has yet to be defined, studies indicate that the formation of multinucleated myotubes from mononucleate myoblasts is dependent on the interaction of β-catenin with a functional cadherin (Redfield *et al.*, 1997).

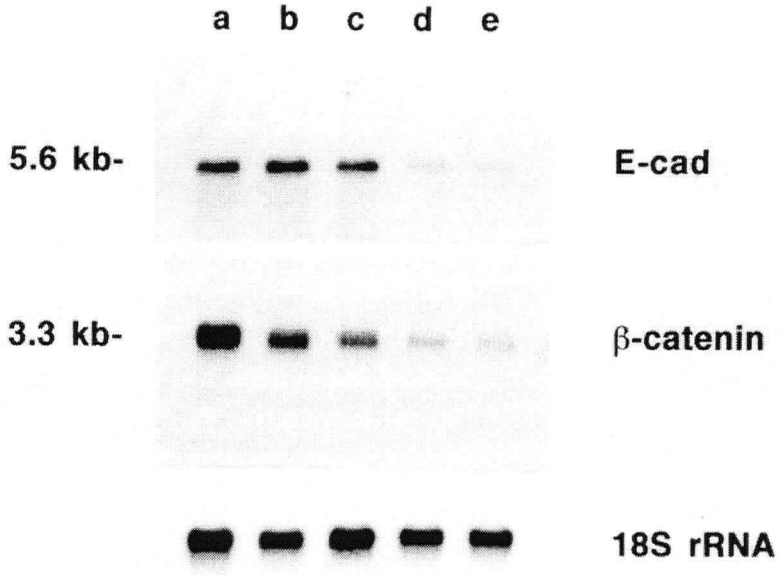
β-catenin also interacts with cad-11, the other cadherin subtype identified in the chorionic villi of the human placenta (MacCalman *et al.*, 1996a). In particular, cad-11/β-catenin complexes have been detected in signet-ring cell carcinomas (Shibata *et al.*, 1996) and invasive breast cancer cell lines (Pishvaian *et al.*, 1999), indicating that this cadherin/catenin complex may promote the neoplastic transformation of epithelial cells. However, cad-11 expression increases during the terminal differentiation and fusion of trophoblastic cells *in vitro* (MacCalman *et al.*, 1996a) and has been localized to the syncytial trophoblast of the human placenta (MacCalman *et al.*, 1996a, 1997b). As cad-11 and β-catenin are differentially expressed during the terminal differentiation and fusion of human trophoblasts *in vitro* and *in vivo* and cad-11/β-catenin complexes do not appear to be present in villous cytotrophoblasts isolated from the human term placenta, it is possible that cad-11 promotes these developmental processes by interacting with other catenins or unidentified cytoplasmic proteins.

In summary, this study demonstrates that β-catenin mRNA concentration and protein expression are down-regulated during the terminal differentiation and fusion of cytotrophoblasts isolated from the human term placenta in a manner similar to E-cad expression. These findings provide the basis for further functional studies of the roles of distinct cadherin/catenin complexes in trophoblast differentiation.

Fig. 2.2.1: (A) Autoradiograms of a Northern blot containing total RNA extracted from villous cytotrophoblasts isolated from human term placentae. The cells were harvested after 0, 12, 24, 48, or 72 h of culture (lanes a-e respectively). This blot was probed for E-cad (upper panel), β-catenin (middle panel), or 18S rRNA (lower panel).

(B) The autoradiograms shown in (A) were scanned using a laser densitometer. The absorbance values obtained for the E-cad and β-catenin mRNA transcripts were normalized to the values obtained for the 18S rRNA. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the 0 h control (mean $\pm$ SEM; n=3; $^{*}P \leq 0.05$ versus 0 h control).

A


a b c d e
5.6 kb-
E-cad
3.3 kb-
β-catenin
18S rRNA

B

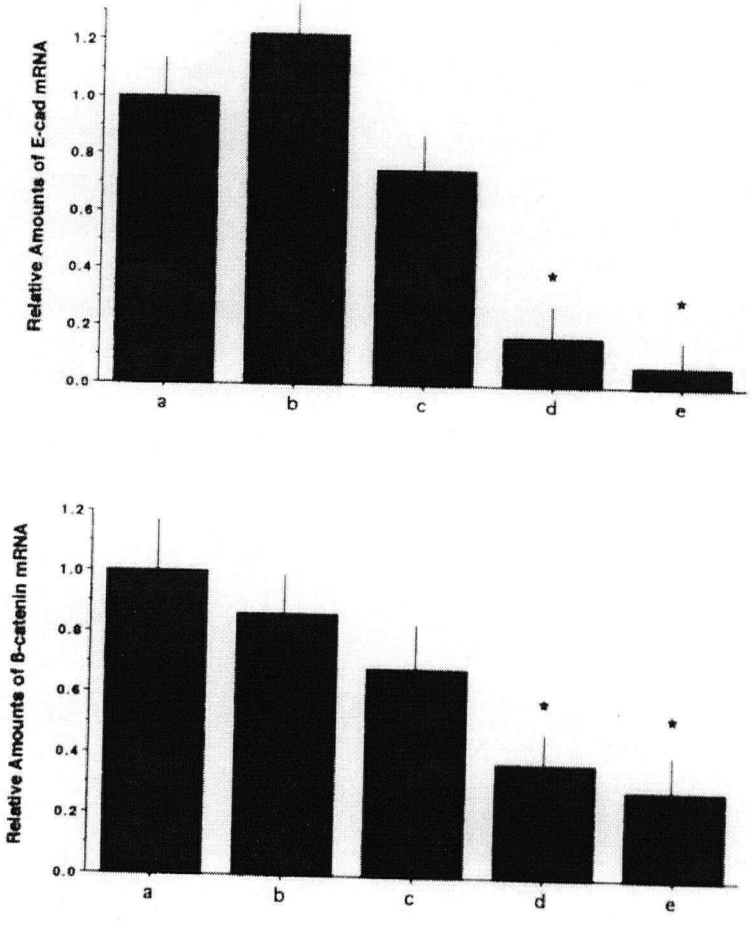
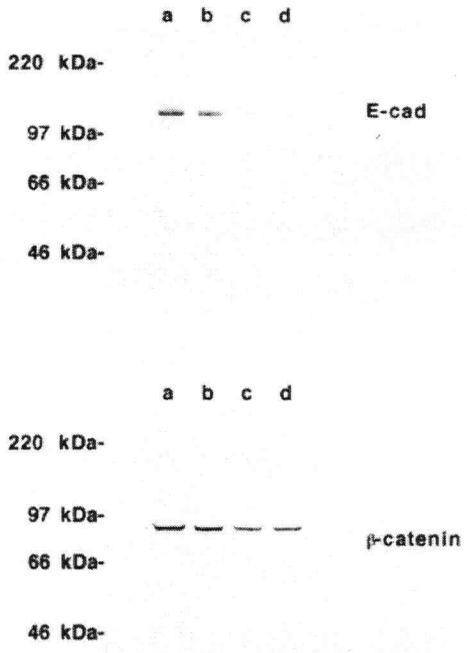

Relative Amounts of E-cad mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
e
Relative Amounts of β-catenin mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
e

Fig. 2.2.2: (A) Western blot analysis of E-cad (upper panel) and β-catenin (lower panel) expression in primary cultures of villous cytotrophoblasts. Protein (20 μg) extracted from villous cytotrophoblasts cultured for 12, 24, 48, or 72h was loaded in each lane (lanes a-d respectively). Western blot analysis was performed using mouse monoclonal antibodies directed against human E-cad or β-catenin. The Amersham ECL system was used to detect antibody bound to antigen. (B) The autoradiograms shown in (A) were scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the 12 h control (mean $\pm$ SEM; n=3; $^{*}P \leq 0.05$ versus 12 h control).

**A**


a b c d
220 kDa-
97 kDa-
66 kDa-
46 kDa-
E-cad
a b c d
220 kDa-
97 kDa-
66 kDa-
46 kDa-
β-catenin

**B**

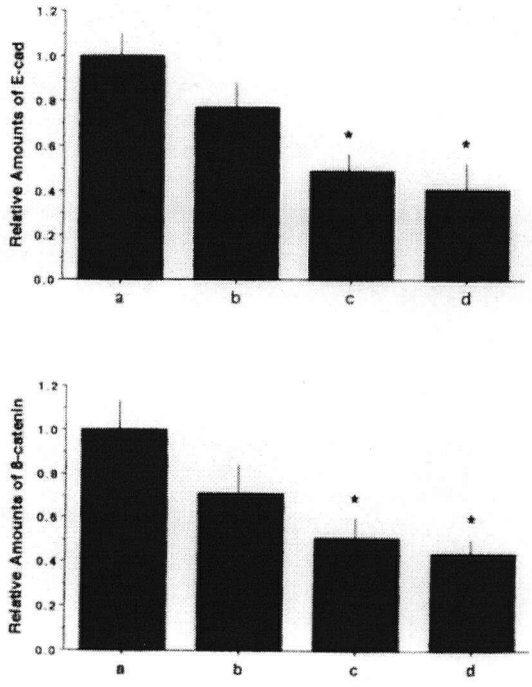
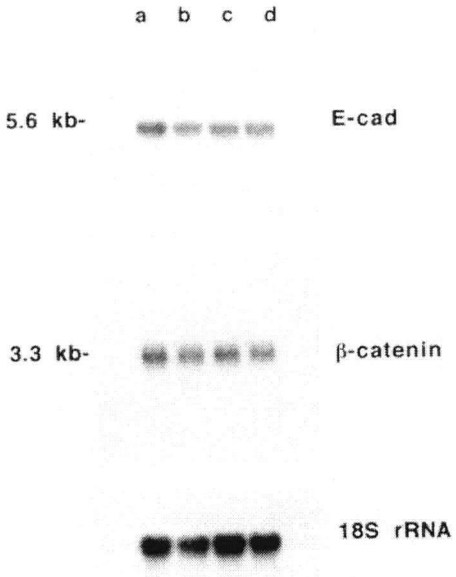

Relative Amounts of E-cad
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
Relative Amounts of β-catenin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d

Fig. 2.2.3: (A) Autoradiograms of a Northern blot containing total RNA extracted from JEG-3 cells cultured for 0, 24, 48, or 72h (lanes a-d respectively). The blot was probed for E-cad (upper panel), β-catenin (middle panel), or 18S rRNA (lower panel).

(B) The autoradiograms shown in (A) were scanned using a laser densitometer. The absorbance values obtained for the E-cad and β-catenin mRNA transcripts were then normalized to the values obtained for the 18S rRNA. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the 0 h control (mean $\pm$ SEM; n=3).

**A**


a b c d
5.6 kb-
E-cad
3.3 kb-
β-catenin
18S rRNA

**B**

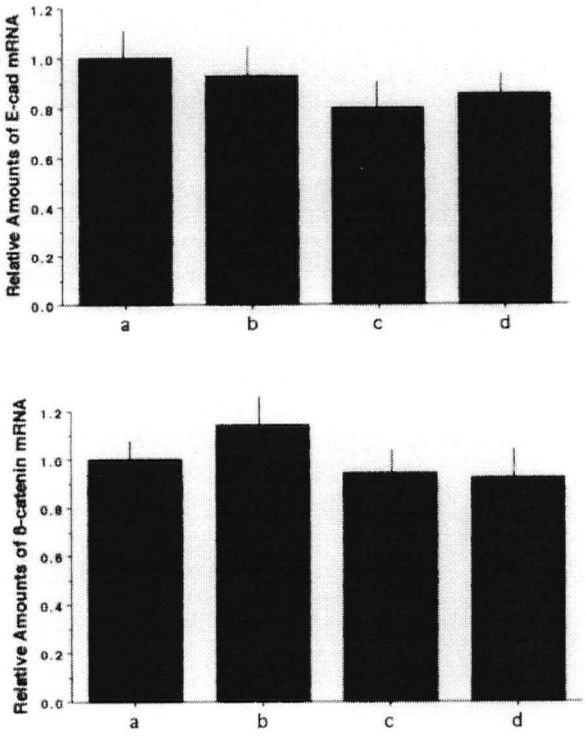
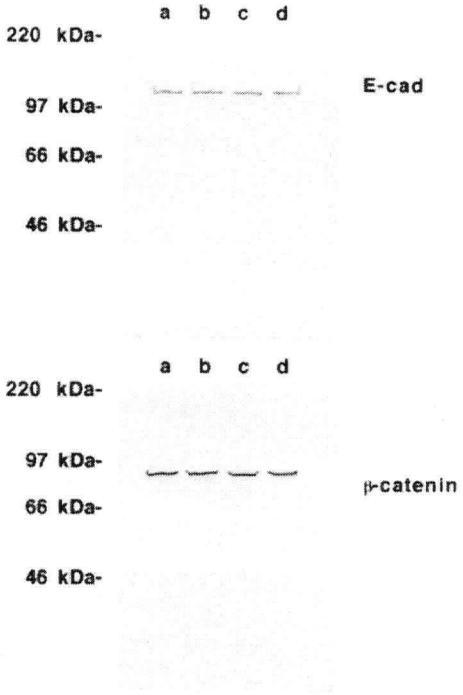

Relative Amounts of E-cad mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
Relative Amounts of β-catenin mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d

Fig. 2.2.4: (A) Western blot analysis of E-cad (upper panel) and β-catenin (lower panel) expression in JEG-3 cells. Protein (20 μg) extracted from JEG-3 cells cultured for 0, 24, 48, or 72 h were loaded in each lane (lanes a-d respectively). Western blot analysis was performed using mouse monoclonal antibodies directed against human E-cad or β-catenin. The Amersham ECL system was used to detect antibody bound to antigen.

(B) The autoradiograms shown in (A) were scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the 0 h control (mean $\pm$ SEM; n=3).

**A**


a b c d
220 kDa-
97 kDa-
66 kDa-
46 kDa-
E-cad
a b c d
220 kDa-
97 kDa-
66 kDa-
46 kDa-
β-catenin

**B**

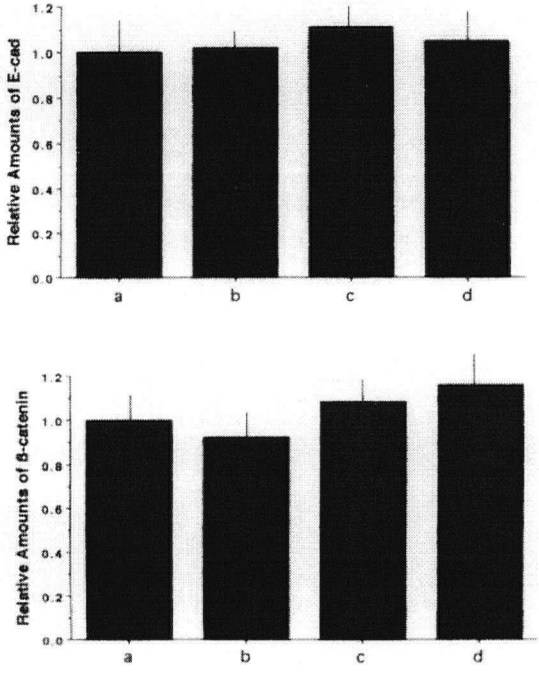

Relative Amounts of E-cad
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
Relative Amounts of β-catenin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d

Fig. 2.2.5: Immunolocalization of E-cad and β-catenin in villous cytotrophoblasts and JEG-3 cells. Villous cytotrophoblasts cultured for 12 (a,b) or 72 h (c,d) were immunostained for E-cad (a,c) or β-catenin (b,d). Similarly, JEG-3 cells cultured for 72 h were immunostained for E-cad (e) and β-catenin (f). Negative controls in which each of the primary antisera were replaced with a non-specific isotype-matched monoclonal antibody are also shown (g,h). Scale bars represents 20 μm.

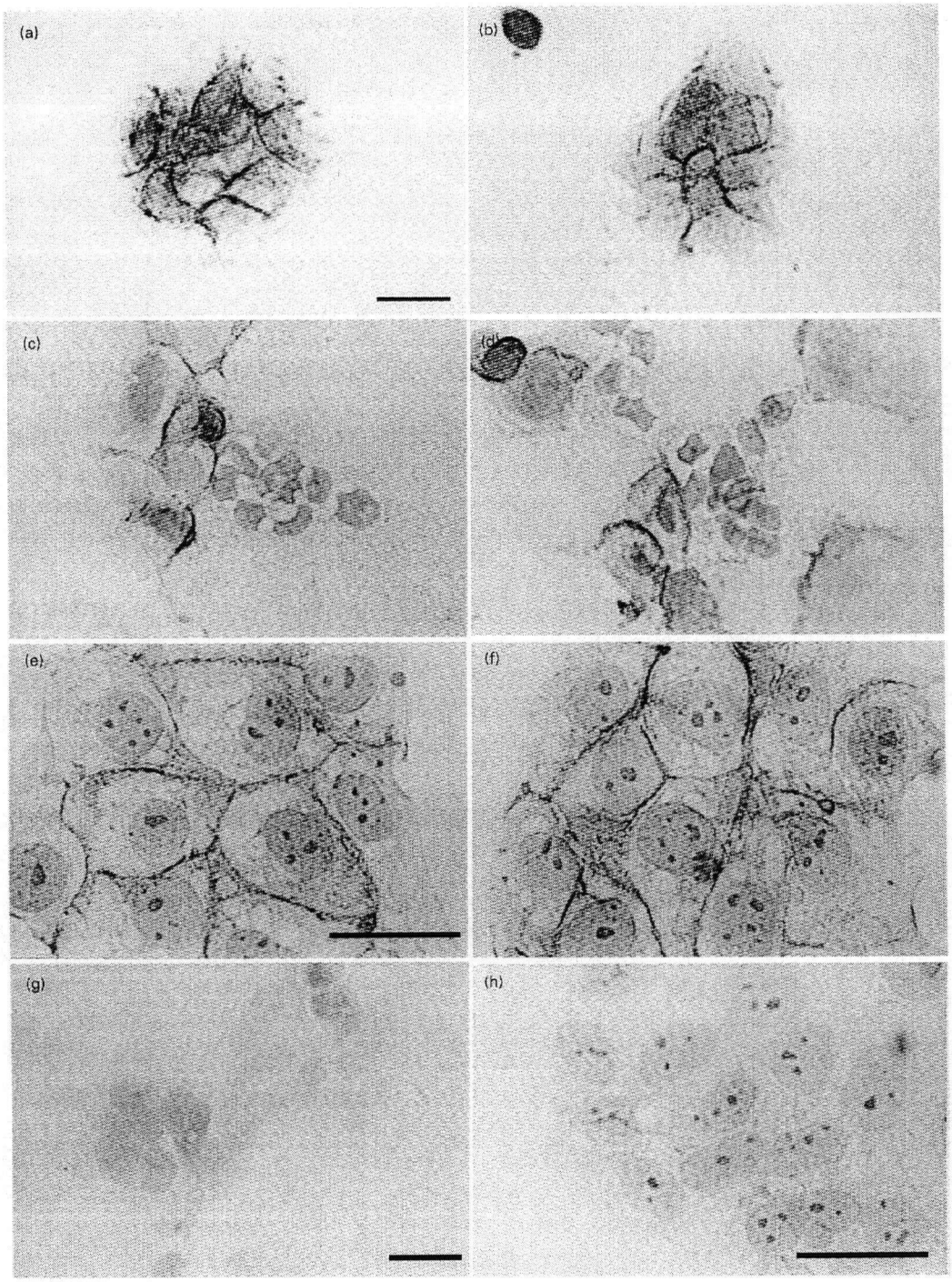

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Fig. 2.2.6: Autoradiogram of an immunoblot containing cell lysates prepared from villous cytotrophoblasts cultured for 48 h and immunoprecipitated with a mouse monoclonal antibody directed against human E-cad (lane a), cad-11 (lane b), or β-catenin (lane c). A negative control in which antiserum was replaced with a non-specific isotype-matched monoclonal antibody is also shown (lane d). The immunoblot was probed with a mouse monoclonal antibody directed against β-catenin. The Amersham ECL system was used to detect antibody bound to antigen.

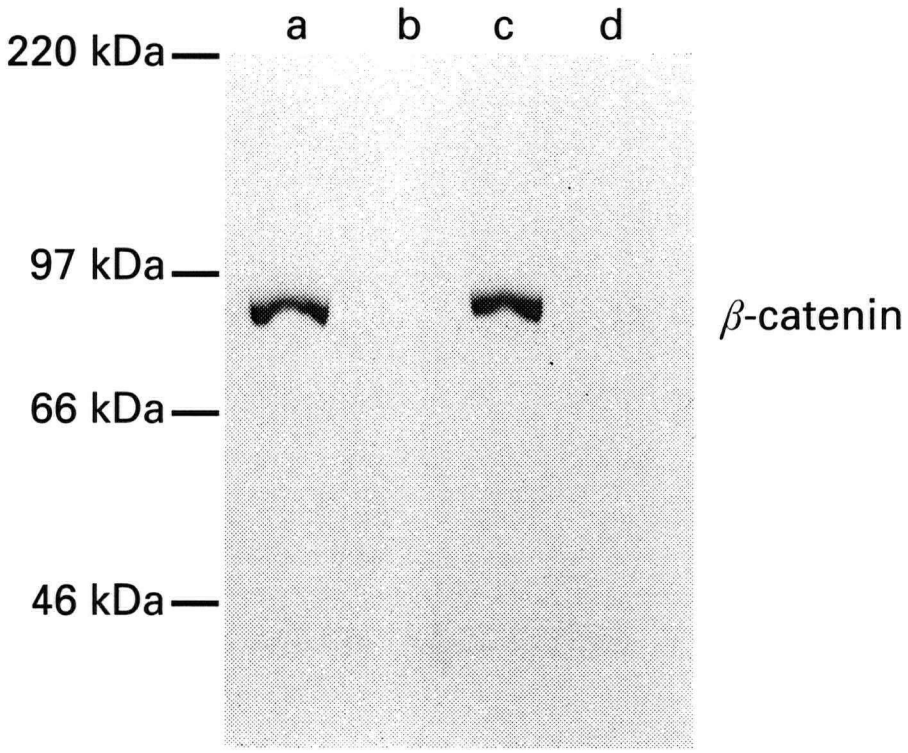
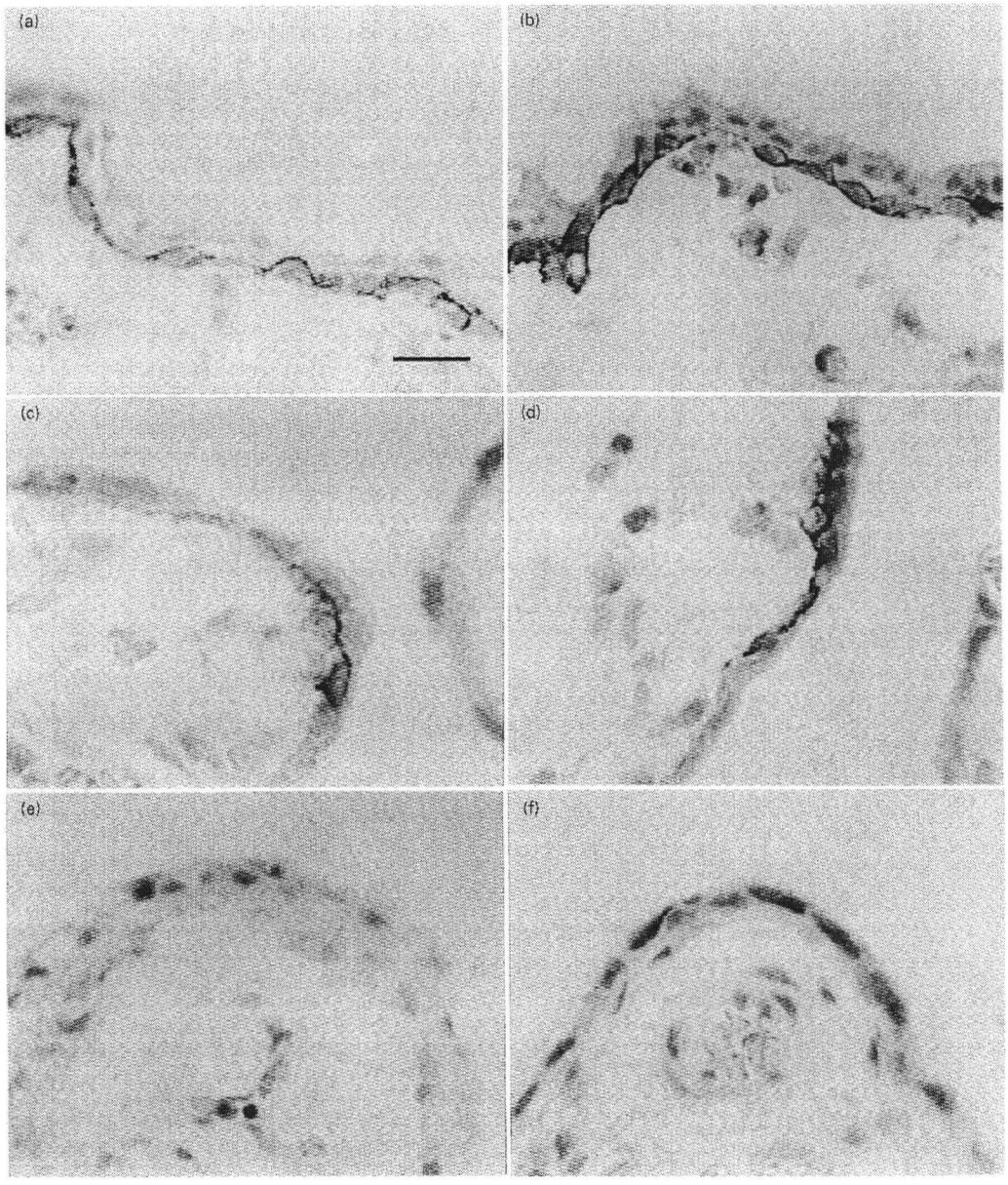

a
b
c
d
220 kDa
97 kDa
66 kDa
46 kDa
β-catenin

Fig 2.2.7. Immunolocalization of E-cad (a,c) and β-catenin (b,d) in the human first trimester (a,b) and term placenta (c,d). Negative controls in which the antisera were replaced with a non-specific isotype-matched monoclonal antibody are also shown (e,f). E-cad and β-catenin immunostaining were detected in the villous cytotrophoblasts but not the syncytial trophoblast of these placental tissues. The scale bar represents 40 μm.


(a)
(b)
(c)
(d)
(e)
(f)

## 2.3: α-, β-, γ-CATENIN, AND p120$^{ctn}$ EXPRESSION DURING THE TERMINAL DIFFERENTIATION AND FUSION OF HUMAN MONONUCLEATE CYTOTROPHOBLASTS *IN VITRO* AND *IN VIVO*


### Abstract

The cadherins play key roles in the formation and organization of the mammalian placenta by mediating cellular interactions and the terminal differentiation of trophoblasts. Although cadherin function is regulated by the cytoplasmic proteins, known as the catenins, the identity and expression pattern(s) of the catenins present in the human placenta have not been characterized. In these studies, we have determined that α-, β-, γ-catenin, and p120$^{ctn}$ expression levels are high in freshly isolated villous cytotrophoblasts but decline as the cells undergo aggregation and fusion to form syncytium with time in culture. In contrast, the expression levels of these four catenin subtypes remained constant in non-fusing JEG-3 choriocarcinoma cells at all of the time points examined in these studies. α-, β-, γ-catenin, and p120$^{ctn}$ expression was further immunolocalized to the mononucleate cells present in these two trophoblastic cell cultures. Similarly, intense immunostaining for all four catenins was detected in the mononucleate villous cytotrophoblasts of the human first trimester placenta. Collectively, these observations demonstrate that the expression levels of α-, β-, γ-catenin, and p120$^{ctn}$ are tightly regulated during the formation of multinucleated syncytium *in vitro* and *in vivo*.


### Introduction

The human placenta is derived from cells of the embryonic trophectoderm (Boyd and Hamilton, 1970). Upon implantation, these trophoblastic cells proliferate and differentiate to

form chorionic villi. The inner trophoblast layer of human chorionic villi is comprised of mitotically active, mononucleate cytotrophoblasts while the outer syncytial trophoblast is a multinucleated cell that is formed by the terminal differentiation and fusion of post-mitotic villous cytotrophoblasts (Richart, 1961; Kliman *et al.*, 1986). The villous cytotrophoblasts form extensive cellular interactions with one another and the syncytial trophoblast (Boyd and Hamilton, 1970; Metz and Weihe, 1980).

Members of the gene superfamily of $Ca^{2+}$-dependent CAMs, known as the cadherins, have been shown to play pivotal roles in trophoblast differentiation and the development of the mammalian placenta (MacCalman *et al.*, 1998). For example, the loss of expression and/or function of E-cad disrupts the formation of a polarized trophectoderm in the murine embryo (Larue *et al.*, 1994; Riethmacher *et al.*, 1995) and inhibits the aggregation of human villous cytotrophoblasts *in vitro* (Coutifaris *et al.*, 1991). Furthermore, antisense oligonucleotides specific for cad-11, the predominant cadherin subtype expressed by the syncytial trophoblast of the human placenta (Chapter 3.1; MacCalman *et al.*, 1996a), are capable of inhibiting the terminal differentiation and fusion of isolated mononucleate cytotrophoblasts (MacCalman, 1997b).

In general, cadherin function is dependent on these CAMs interacting with the cytoplasmic proteins known as the catenins (Aberle *et al.*, 1996). To date, the best characterized catenins are α-, β-, and γ-catenin. β-catenin and γ-catenin interact with the cadherins in a mutually exclusive manner (Butz and Kemler, 1994; Nathke *et al.*, 1994), whereas α-catenin links both cadherin-catenin complexes to the actin-based cytoskeleton by direct interaction (Rimm *et al.*, 1995a) or via α-actinin (Knudsen *et al.*, 1995). In addition, $p120^{ctn}$, a cytoplasmic protein that is distantly related to β- and γ-catenin, has recently been shown to interact with the cadherins (Reynolds *et al.*, 1994; Ohkubo and Ozawa, 1999). However, $p120^{ctn}$ does not bind α-

catenin and is therefore, believed not to link the cadherins to the cytoskeleton (Daniel and Reynolds, 1995). Although the biological function(s) of $p120^{ctn}$ remain poorly understood, it has been suggested that this cytoplasmic protein modulates cadherin-mediated cellular interactions and/or plays a key role in the intracellular signaling events mediated by the distinct cadherin-catenin complexes during development.

The formation and localization of cadherin complexes containing either β- or γ-catenin appears to be differentially regulated during cellular differentiation and development. In particular, complexes containing β-catenin are more abundant in poorly differentiated cells whereas γ-catenin complexes increase with cellular differentiation (Butz and Larue, 1995; Lampugnani *et al.*, 1995). Although β-catenin has been co-localized with E-cad in the inner cell mass and trophectoderm of murine (Ohsugi *et al.*, 1996) and bovine embryos (Barcroft *et al.*, 1998) and in primary cultures of human villous cytotrophoblasts (Chapter 2.2; Getsios *et al.*, 2000), the expression pattern(s) of the other catenin subtypes during the morphological differentiation of mononucleate cytotrophoblasts into syncytium has not been characterized.

As a first step in addressing these outstanding issues, we have examined α-, β-, γ-catenin, and $p120^{ctn}$ expression levels in primary cultures of villous cytotrophoblasts undergoing aggregation, differentiation, and fusion *in vitro* and in cultures of JEG-3 cells, a choriocarcinoma cell line which is not fusion-competent under standard culture conditions, using Western blot analysis. The cellular distribution of α-, β-, γ-catenin, and $p120^{ctn}$ in these trophoblastic cell cultures was also determined by immunocytochemistry. Finally, we have immunolocalized these four catenins in human first trimester placental tissues.

## Materials and Methods

*Tissues*

First trimester (approximately 12 weeks of gestation; n=3) and term placental tissues (n=6) were obtained from women undergoing elective termination of pregnancy and cesarean section, respectively. Research on human placental tissues was approved by the Committee for Ethical Review of Research Involving Human Subjects, University of British Columbia, Vancouver, Canada. All patients provided informed consent for these studies.

*Cell preparation and culture*

Villous cytotrophoblasts were prepared from human term placentae as previously described by Kliman *et al.* (1986). This method, which utilizes serial trypsin-DNAse digestions to purify cells, yields a highly enriched preparation of cytotrophoblasts. Following isolation, these cells were cultured in DMEM containing 25 mM glucose, 25 mM HEPES, and 50 μg/ml gentamicin and supplemented with 10% heat-inactivated FCS.

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) were maintained in the culture medium described above.

Villous cytotrophoblasts ($1.2 \times 10^7$ cells) or JEG-3 cells ($5 \times 10^6$ cells) were plated in 100 mm culture dishes and cultured for 12, 24, 48, or 72 h before being harvested for Western blot analysis. Villous cytotrophoblasts ($5 \times 10^5$ cells) or JEG-3 cells ($2 \times 10^5$ cells) were also seeded on glass coverslips (2 x 2 cm) and processed for immunocytochemical analysis after 72 h of culture.

*Western Blot Analysis*

For Western blot analysis, cultures of villous cytotrophoblasts or JEG-3 cells were washed twice with PBS and incubated in 1 ml of chilled cell lysis buffer (Tris-HCl, pH 7.5 containing 0.5% NP-40, 0.5 mM $CaCl_2$ and 1.0 mM PMSF) at 4 °C for 30 min on a rocking platform. The cell lysates were centrifuged at 10,000 x g for 20 min and the supernatant used in the Western blot analysis. Protein concentration in the cellular extracts was determined using the BCA kit (Pierce Chemicals, Rockford, IL). Aliquots (20 μg) were subjected to SDS-PAGE under reducing conditions, as described by Laemmli (1970). The stacking gels contained 5% acrylamide and the separating gels were composed of 7.5% acrylamide. The proteins were electrophoretically transferred from the gels onto nitrocellulose paper according to the procedures of Towbin *et al.* (1979). The nitrocellulose blots were probed with mouse monoclonal antibodies directed against human α-, β-, γ-catenin, or $p120^{ctn}$ (Transduction Laboratories, Lexington, KY). To confirm that the villous cytotrophoblasts were undergoing terminal differentiation and fusion *in vitro*, blots containing total protein extracted from these cell cultures were also probed with monoclonal antibodies directed against human E-cad (Transduction Labs) or cad-11 (C11-113H; ICOS Corp, Bothell, WA). The Amersham ECL system (Amersham Life Science Inc, Oakville, ON) was used to detect antibody bound to antigen. The autoradiograms were then scanned using a laser densitometer.

*Immunolocalization of α-, β-, γ-catenin, or $p120^{ctn}$ in trophoblastic cell cultures and human placental tissues.*

Villous cytotrophoblasts or JEG-3 cells were washed three times in PBS and fixed in 4% paraformaldehyde for 15 min at RT. The cells were then rinsed twice with PBS and stored at 4 °C until being used in these studies.

Frozen sections (8 μm) were prepared from human first trimester placental tissues that had been snap-frozen in the embedding compound, OCT (Miles Inc., Elkhart, IN). The sections were fixed with 4% paraformaldehyde for 15 min at RT and washed twice with PBS prior to being immunostained.

The fixed cells and tissue sections were immunostained using mouse monoclonal antibodies directed against human α- (Santa Cruz Biotech. Inc., Santa Cruz, CA), β-, γ-catenin, or $p120^{ctn}$ (Transduction Labs). Sequential incubations were performed according to the methods of Cartun and Pedersen (1989) and included 10% normal horse serum for 30 min, primary antibody at 37 °C for 1h, secondary biotinylated antibody at 37 °C for 45 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37 °C for 30 min, and three washes (5 min each) in PBS. The cells and tissue sections were then exposed to chromagen reaction solution (0.035% diaminobenzidine and 0.03% $H_2O_2$) for 10 min, washed in tap water for 5 min, counterstained in haematoxylin, dehydrated, cleared, and mounted.

*Statistical Analysis*

The results for the Western blot analysis are presented as the mean relative absorbance (± SEM) for three independent experiments. Statistical difference between time points was assessed by ANOVA. Differences were considered significant for $P \leq 0.05$. Significant difference between the means was determined using the least significant difference test.

**Results**

Western blot analysis revealed that protein species corresponding to α- (102 kDa), β- (92 kDa), γ-catenin (82 kDa), and $p120^{ctn}$ (120 kDa) were present in all of the cellular extracts prepared

from the villous cytotrophoblast or JEG-3 cell cultures. Maximum levels of these four catenin protein species were detected in primary cultures of villous cytotrophoblasts at 12 h (Fig. 2.3.1). There was a significant decline in α-, β-, γ-catenin, and $p120^{ctn}$ expression after 48 h of culture. The expression levels of these four catenins continued to decrease until the termination of these studies at 72 h. The down-regulation of α-, β-, γ-catenin, and $p120^{ctn}$ correlated with the terminal differentiation and fusion of isolated villous cytotrophoblasts, as determined by a decrease in E-cad expression and a concomitant increase in the expression levels of the 125 kDa cad-11 protein species present in these cell cultures (MacCalman *et al.*, 1996a). In contrast, there was no significant difference in the expression levels of these four catenin subtypes in cultures of JEG-3 cells at any of the time points examined in these studies (Fig. 2.3.2).

α-, β-, γ-catenin, and $p120^{ctn}$ expression was immunolocalized to the villous cytotrophoblasts undergoing aggregation with the multinucleated syncytium and the mononucleate cells that had migrated on top of the flattened cytoplasmic expanse of these syncytial structures at 72 h (Fig. 2.3.3). In contrast, we failed to detect immunostaining for these four catenins on the surface of the large multinucleated syncytium which formed in these primary cell cultures, with the exception of areas that were in direct contact with unfused cytotrophoblasts. α-, β-, γ-catenin, and $p120^{ctn}$ immunostaining was readily detectable in non-fusing JEG-3 cells cultured until the termination of these studies at 72 h (Fig. 2.3.4).

Finally, α-, β-, γ-catenin, and $p120^{ctn}$ expression was immunolocalized to the intercellular borders between mononucleate villous cytotrophoblasts and the syncytial trophoblast of the human first trimester placenta (Fig. 2.3.5). In contrast, significant expression of these four catenin subtypes was not detected in the outer syncytial trophoblast layer of these placental tissues.

**Discussion**

Villous cytotrophoblasts interact with one another through the assembly of intercellular junctions at areas of contact between adjacent cells *in vitro* and *in vivo* (Metz and Weihe, 1980; Douglas and King, 1990; Coutifaris *et al.*, 1991). These junctional complexes are subsequently disassembled as the cytotrophoblasts undergo terminal differentiation and fusion to form the multinucleated syncytial trophoblast. E-cad, in association with β-catenin, is believed to mediate the formation of adherens, gap, and tight junctions between adjacent epithelial cells (Gumbiner *et al.*, 1988; Marrs *et al.*, 1995; Troxell *et al.*, 1999). γ-catenin has also been associated with junctional complex formation (Lewis *et al.*, 1997) and is present in both adherens-like and desmosomal junctions of epithelial cells (Knudsen and Wheelock, 1992; Troyanovsky *et al.*, 1994b). In these studies, we have determined that α-, β-, γ-catenin, and $p120^{ctn}$ expression is high in freshly isolated villous cytotrophoblasts and decline as these cells undergo aggregation and fusion to form syncytium *in vitro*. In contrast, the expression of these four catenin subtypes was maintained in non-fusing JEG-3 cell cultures. This expression pattern correlates with those previously observed for E-cad in these trophoblastic cell cultures (Coutifaris *et al.*, 1991; Rebut-Bonneton *et al.*, 1993; MacCalman *et al.*, 1996a). Collectively, these observations suggest that a reduction in the expression levels of α-, β-, γ-catenin, and $p120^{ctn}$, in association with E-cad, results in the loss of integrity and/or organization of intercellular junctions during the terminal differentiation and fusion of human villous cytotrophoblasts.

β-catenin null mutant mice die at early stages of development (Haegel *et al.*, 1995) whereas γ-catenin gene knock-out embryos progress through the early developmental stages but subsequently die as a result of aberrant cardiac development (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996). Although β- and γ-catenin are believed to play important roles in the formation and

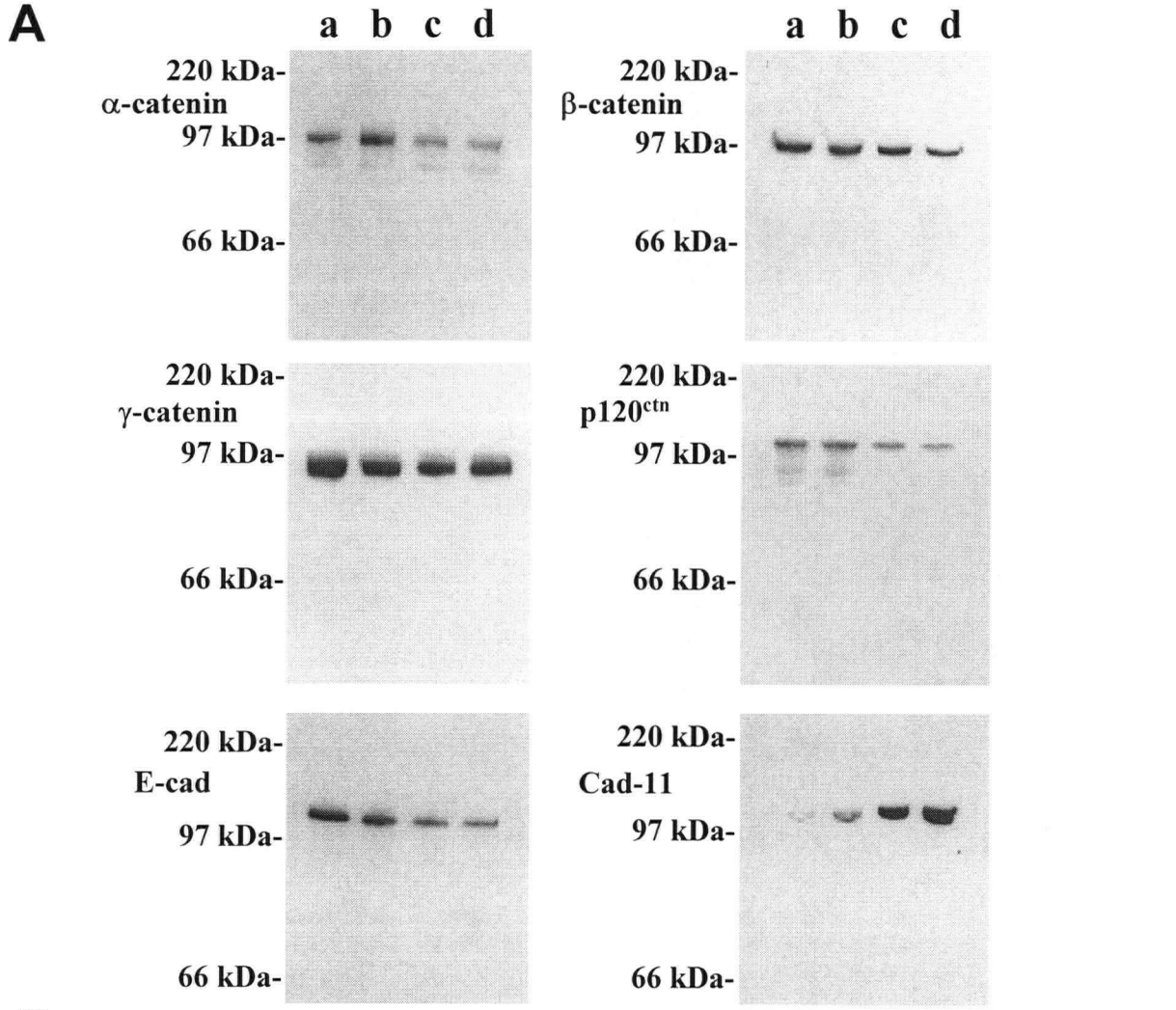
organization of the mammalian placenta, differentiation of the trophectoderm does not appear to be affected in either of these null mutant mice. The ability of the β-catenin gene knock-out mice to form a polarized trophectoderm has been attributed to the persistence of maternally-derived gene products. However, instead of being restricted to desmosomes, γ-catenin was co-localized with E-cad in the trophectodermal layer of these null mutant mice (Ohsugi *et al.*, 1997). Taken together, these observations suggest that γ-catenin can be substituted for β-catenin in these cells, thereby promoting E-cad-mediated cellular interactions during the formation of the trophectoderm. To date, the distinct role(s) of β- and γ-catenin in human placentation have not been defined. However, the regulated expression of these catenins during the terminal differentiation and fusion of villous cytotrophoblasts suggests that β- and γ-catenin, alone or in combination, are involved in the cellular events that are mediated by E-cad during this developmental process.

A switch between E-cad and cad-11 expression during the formation of mesodermal cell layers in mouse embryos was not associated with alterations in the expression levels and/or distribution of α- or β-catenin in these cells (Butz and Larue, 1995; Hoffmann and Balling, 1995). The terminal differentiation and fusion of villous cytotrophoblasts has also been associated with a reduction in E-cad and a concomitant increase in cad-11 expression levels (MacCalman *et al.*, 1996a). As cad-11 and the four catenin subtypes examined in these studies are differentially expressed during the morphological differentiation of human trophoblasts *in vitro* and *in vivo*, it is tempting to speculate that cad-11 promotes these developmental processes by interacting with other catenins and/or unidentified cytoplasmic proteins. In support of this hypothesis, Kuch *et al.* (1997) observed a marked decrease in the levels of the cadherin/catenin complexes identified in cultures of mononucleate myoblasts undergoing terminal differentiation and fusion to form multinucleated myotubes.

In conclusion, we have demonstrated that α-, β-, γ-catenin, and $p120^{ctn}$ expression levels are down-regulated during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro* and *in vivo*. These studies not only add to our understanding of the adhesive mechanisms operative during the formation and organization of the human placenta but provide the basis for further functional studies examining the role(s) of distinct cadherin/catenin complexes in cellular differentiation.

Fig. 2.3.1: (A) Western blot analysis of α-, β-, γ-catenin, or $p120^{ctn}$ expression in primary cultures of villous cytotrophoblasts. Protein (20 μg) extracted from cytotrophoblasts cultured for 12, 24, 48, or 72 h were loaded in each lane (lanes a-d, respectively). Western blot analysis was performed using mouse monoclonal antibodies directed against α-, β-, γ-catenin, or $p120^{ctn}$. To confirm that these cells were undergoing terminal differentiation and fusion *in vitro*, these blots were also probed using monoclonal antibodies directed against E-cad and cad-11. The Amersham ECL system was used to detect antibody bound to antigen. The positions of the molecular weight standards are shown on the left hand side of the panels.

(B) The autoradiograms were scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the mean obtained for the respective catenin protein species at 12 h and are represented in the bar graphs (mean $\pm$ SEM; *$P \leq 0.05$).


A
a b c d
220 kDa-
α-catenin
97 kDa-
66 kDa-
220 kDa-
β-catenin
97 kDa-
66 kDa-
220 kDa-
γ-catenin
97 kDa-
66 kDa-
220 kDa-
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Cad-11
97 kDa-
66 kDa-

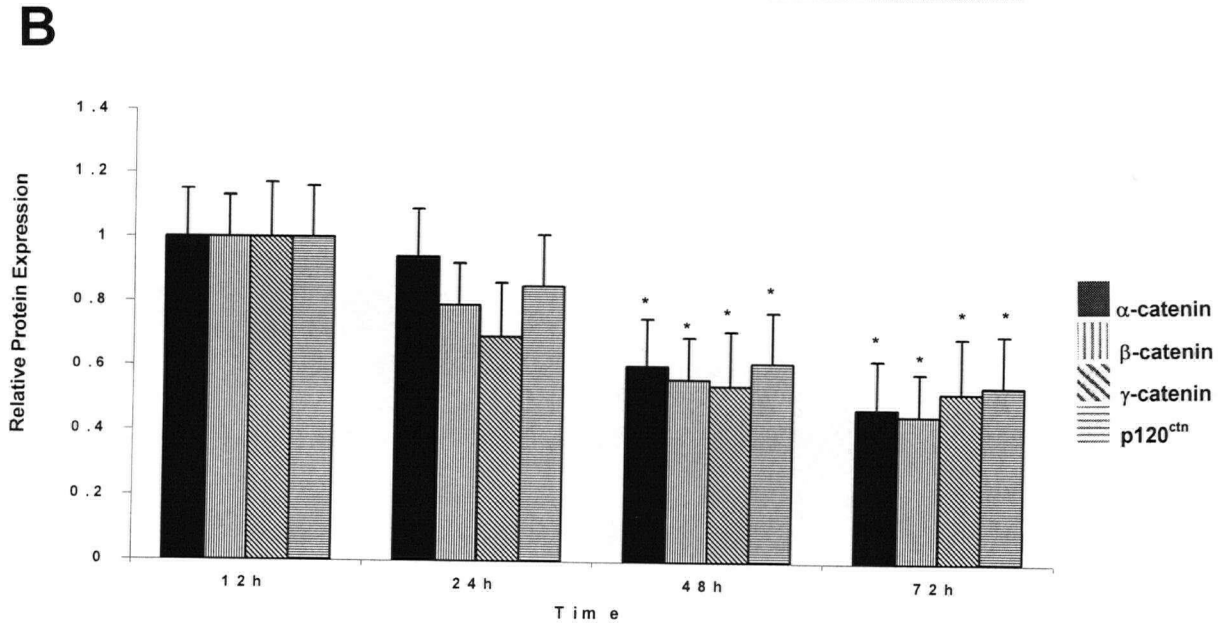
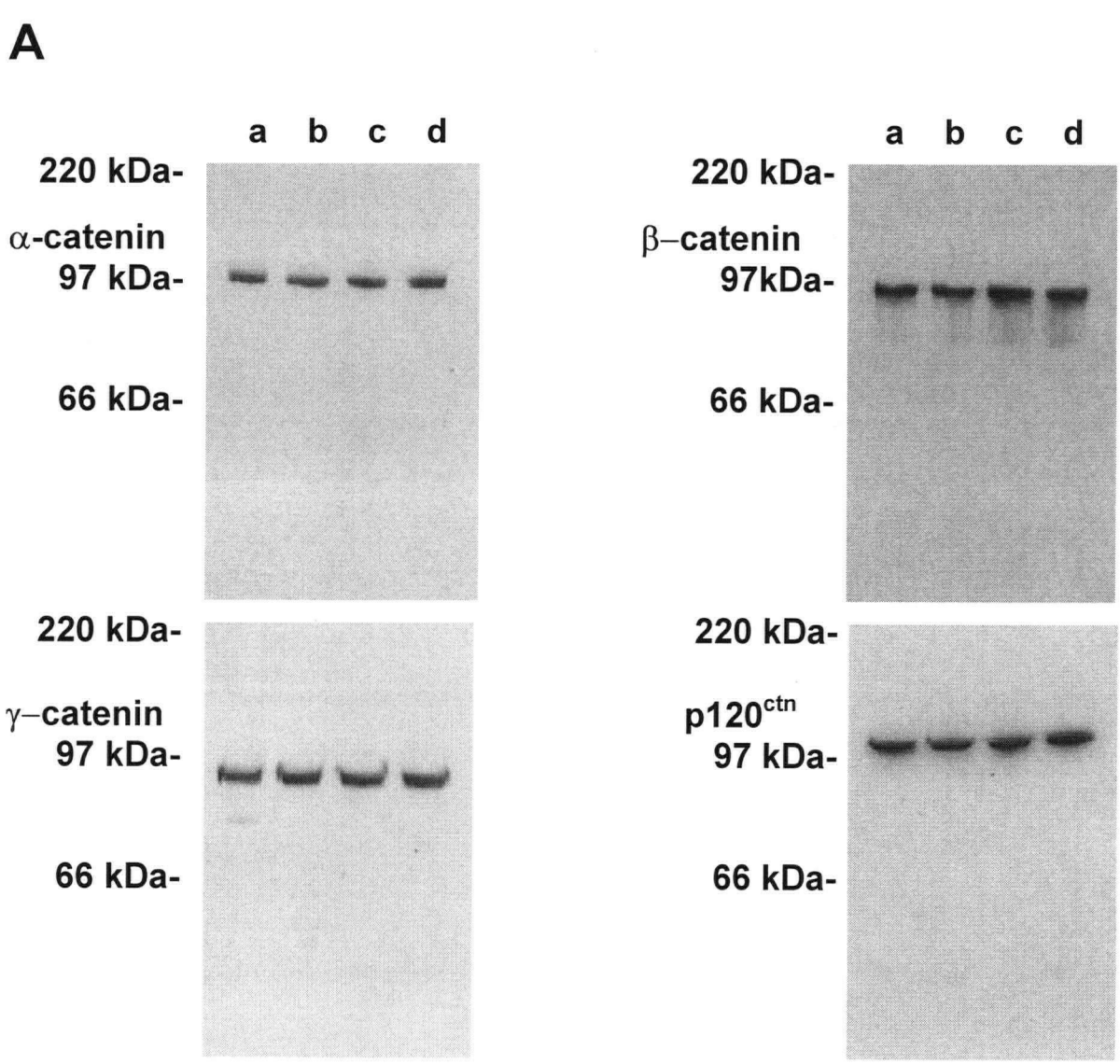

B
Relative Protein Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
12h
24h
48h
72h
Time
α-catenin
β-catenin
γ-catenin
p120ctn

Fig. 2.3.2: (A) Western blot analysis of α-, β-, γ-catenin, or $p120^{ctn}$ expression levels in JEG-3 cells. Protein (20 μg) extracted from JEG-3 cells cultured for 12, 24, 48, or 72 h were loaded in each lane (lanes a-d, respectively). Western blot analysis was performed using mouse monoclonal antibodies directed against α-, β-, γ-catenin, or $p120^{ctn}$. The Amersham ECL system was used to detect antibody bound to antigen. The positions of the molecular weight standards are shown on the left hand side of the panels.

(B) The autoradiograms were scanned using a laser densitometer. The results derived from this analysis, as well as from two other studies (autoradiograms not shown), were standardized to the mean obtained for the respective catenin protein species at 12 h and are represented in the bar graphs (mean ± SEM).


A
a b c d
220 kDa-
α-catenin
97 kDa-
66 kDa-
a b c d
220 kDa-
β–catenin
97kDa-
66 kDa-
220 kDa-
γ–catenin
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p120ctn
97 kDa-
66 kDa-

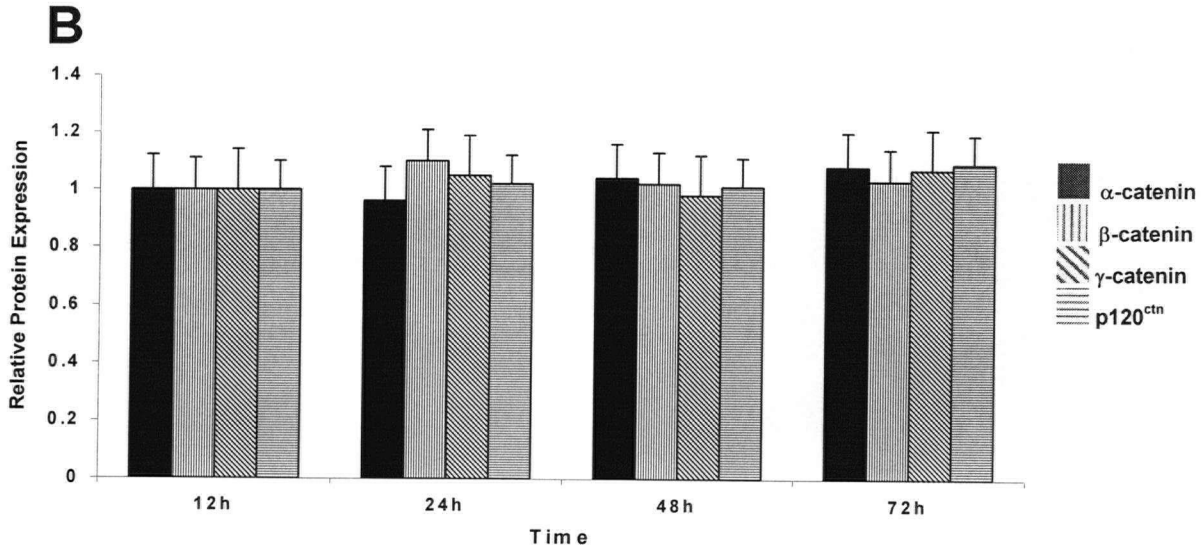

B
Relative Protein Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
12h
24h
48h
72h
Time
α-catenin
β-catenin
γ-catenin
p120ctn

Fig. 2.3.3: Immunolocalization of α-, β-, γ-catenin, or $p120^{ctn}$ in primary cultures of villous cytotrophoblasts. α-, β-, γ-catenin, or $p120^{ctn}$ (panels A-D, respectively) were localized to the mononucleate cytotrophoblasts (arrows) but not the multinucleated syncytium present in these primary cell cultures at 72 h. Negative controls in which the primary antibody was omitted or replaced with a non-specific isotype-matched monoclonal antibody are also shown (panel E and F, respectively). The scale bar represents 20 μm.

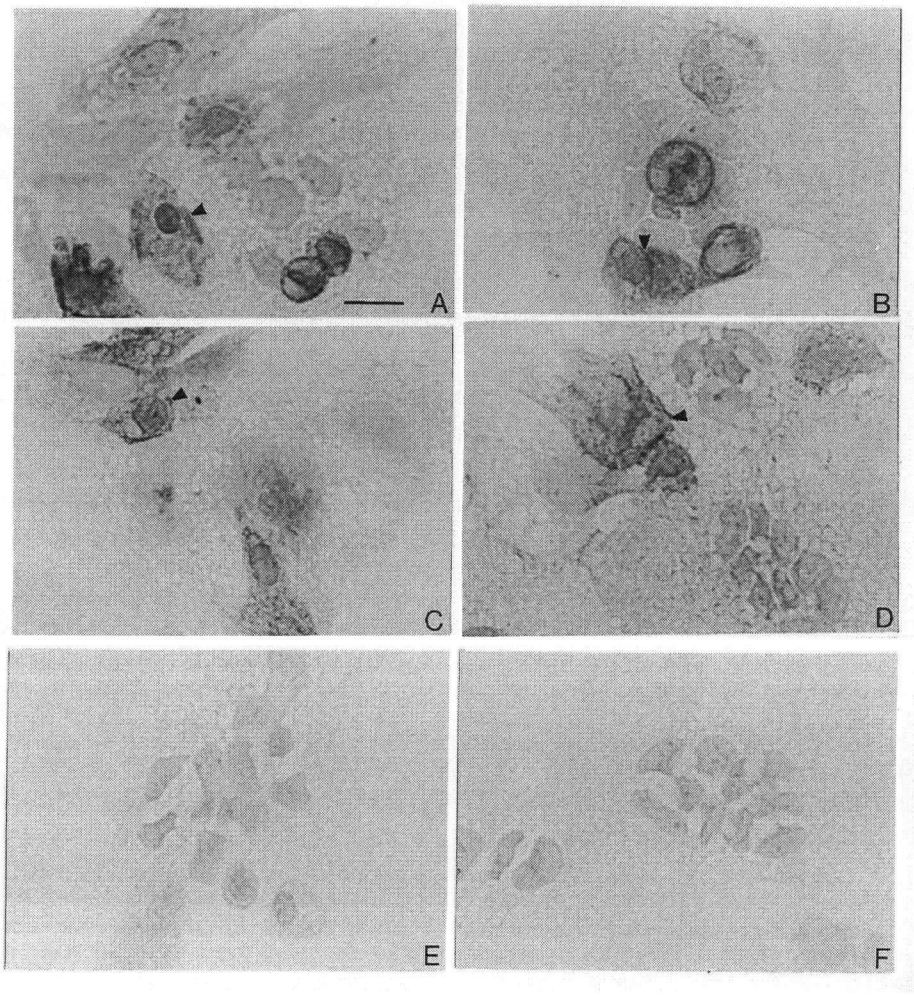

A
B
C
D
E
F

Fig. 2.3.4: Immunolocalization of α-, β-, γ-catenin, or $p120^{ctn}$ in JEG-3 cells. JEG-3 cells cultured for 72 h were immunostained for α-, β-, γ-catenin, or $p120^{ctn}$ (panels A-D, respectively). Negative controls in which the primary antibody was omitted or replaced with a non-specific isotype-matched monoclonal antibody are also shown (panel E and F, respectively). The scale bar represents 10 μm.

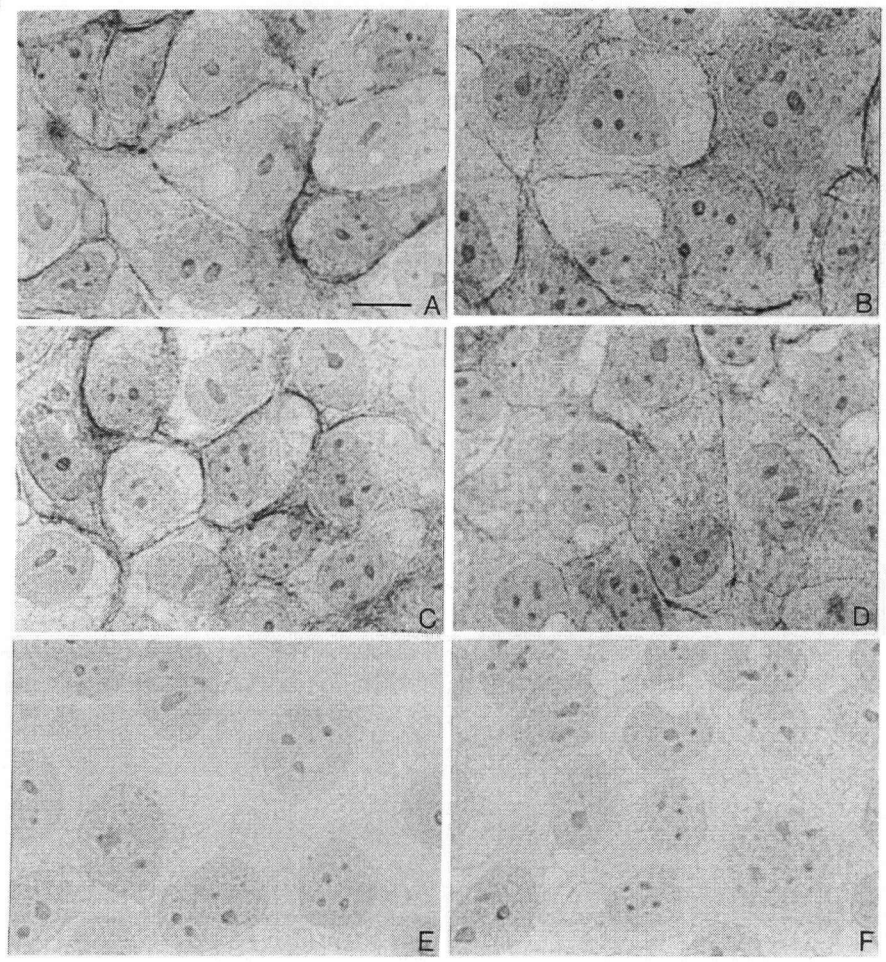
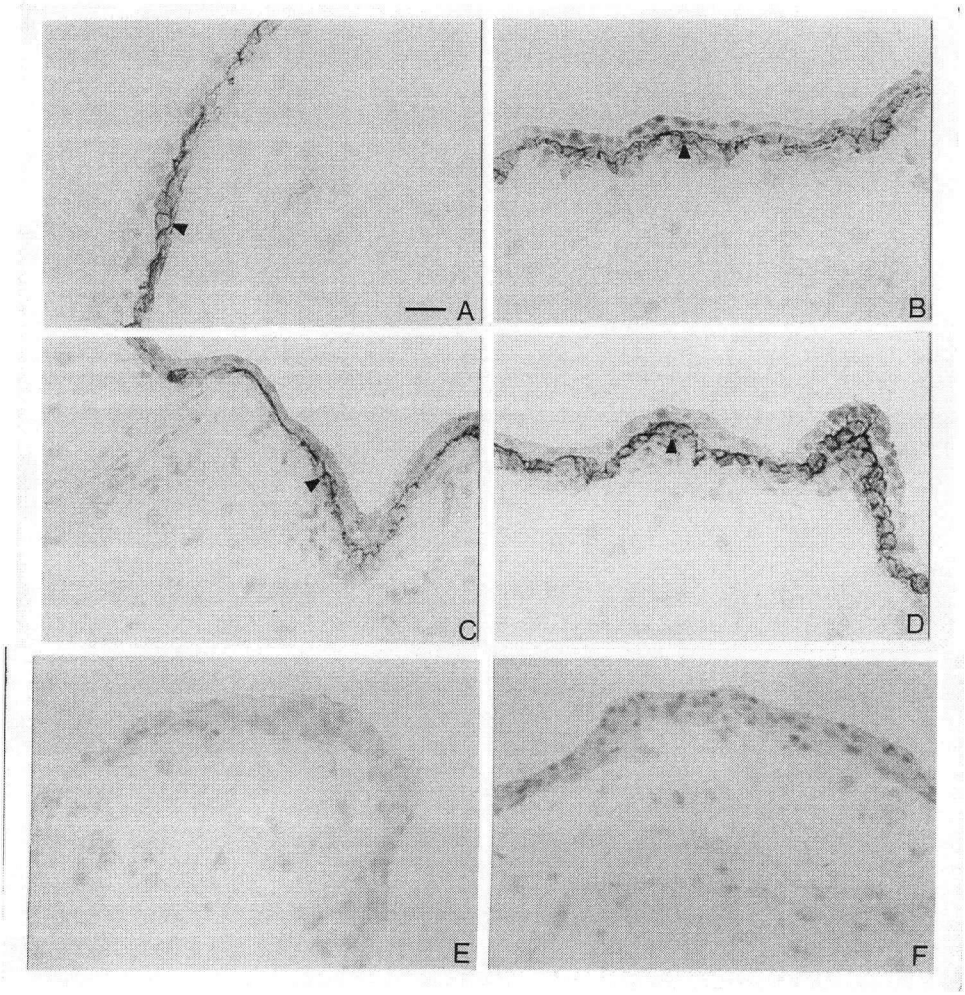

A
B
C
D
E
F

Fig. 2.3.5: Immunolocalization of α-, β-, γ-catenin, or $p120^{ctn}$ in the villous cytotrophoblasts (arrows) of the human first trimester placenta (panels A-D, respectively). Negative controls in which the primary antiserum was omitted or replaced with a non-specific isotype-matched monoclonal antibody are also shown (panel E and F, respectively). The scale bar represents 20 μm.


A
B
C
D
E
F

# CHAPTER III: STUDIES EXAMINING THE ROLE OF CADHERIN-11 IN HUMAN TROPHOBLAST DIFFERENTIATION AND FUSION *IN VITRO*

## 3.1: Preface

In this chapter, we examined the ability of cad-11 to mediate the formation of multinucleated syncytium from mononucleate trophoblastic cells *in vitro*. To define the role(s) of cad-11 in this developmental process, we used two complementary experimental approaches. Firstly, we examined the effects of ectopic cad-11 expression, using a mammalian expression vector containing a full-length cad-11 cDNA, on the morphological and functional differentiation of non-fusing JEG-3 choriocarcinoma cells. In preliminary studies, we determined that >70% of JEG-3 cells were transfected with a similar expression vector containing the β-galactosidase gene, using 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) cytochemistry (Appendix II). Ectopic cad-11 expression in JEG-3 cells was associated with a loss of endogenous E-cad and β-catenin expression, a reduction in cellular proliferation, an increase in the levels of the mRNA transcript that encodes βhCG, and the formation of multinucleated syncytium in these cultures. Cad-11 was subsequently immunolocalized to the multinucleated cells that formed in these cultures following transfection with this mammalian expression vector. We next examined the ability of antisense oligonucleotides, specific for cad-11, to modulate the formation of multinucleated syncytium in villous cytotrophoblasts isolated from the human term placenta. Villous cytotrophoblasts cultured in the presence of these antisense oligonucleotides were capable of forming large cellular aggregates but failed to undergo terminal differentiation and fusion with time in culture, as determined by E-cad immunostaining.

Taken together, these observations demonstrate that cad-11 plays a key role in the morphological and functional differentiation of human villous cytotrophoblasts *in vitro* and

provide the first demonstration of a biological function for this recently characterized type 2 classical cadherin subtype during cellular differentiation. The results of these studies are presented in a manuscript entitled "Cadherin-11 expression levels modulate the terminal differentiation and fusion of human trophoblastic cells *in vitro*" that has been submitted for publication to the *Journal of Cell Science* (S Getsios and CD MacCalman).

The antisense oligonucleotide experiments and the immunolocalization experiments following the transfection of a full-length cad-11 cDNA into JEG-3 cells were performed by Dr. CD MacCalman. The remainder of the research was conducted by S Getsios under the supervision of Dr. CD MacCalman.

## 3.2: CADHERIN-11 EXPRESSION LEVELS MODULATE THE TERMINAL DIFFERENTIATION AND FUSION OF HUMAN TROPHOBLASTIC CELLS *IN VITRO*


### Abstract

A switch in the expression of the cadherin gene superfamily of CAMs mediates tissue morphogenesis. We have recently determined that the two cadherin subtypes, E-cad and cad-11, are differentially expressed in primary cultures of villous cytotrophoblasts isolated from the human term placenta. E-cad expression levels are highest in villous cytotrophoblasts and decrease as these mononucleate cells undergo terminal differentiation and fusion with time in culture. In contrast, cad-11 expression levels increase during the formation of multinucleated syncytium in these trophoblastic cell cultures. To define the role(s) of cad-11 in this developmental process, we have examined the effects of ectopic cad-11 expression on cellular differentiation and fusion in the mononucleate trophoblastic cell line, JEG-3 choriocarcinoma cells. The transfection of a mammalian expression vector containing a full-length cad-11 cDNA into JEG-3 cells resulted in a loss of E-cad expression and the formation of multinucleated syncytium with time in culture. Cad-11 expression in these cells also reduced cellular proliferation and increased the levels of the mRNA transcript encoding βhCG, a biochemical marker of trophoblast differentiation. The ability of antisense oligonucleotides specific for cad-11 to inhibit the terminal differentiation and fusion of primary villous cytotrophoblast cultures was also examined. Cytotrophoblasts cultured in the presence of these antisense oligonucleotides failed to up-regulate cad-11 expression but continued to express E-cad with time in culture. Although the villous cytotrophoblasts were capable of forming large cellular aggregates, these cells did not undergo terminal differentiation and fusion, as determined by E-cad

immunostaining. Collectively, these observations demonstrate that cad-11 plays a pivotal role in the morphological and functional differentiation of mononucleate trophoblastic cells *in vitro*.

## Introduction

The cadherins are a gene superfamily of integral membrane glycoproteins that mediate $Ca^{2+}$-dependent cell adhesion in a homophilic manner (Takeichi, 1995). This gene superfamily has been divided into at least two distinct subfamilies: type 1 and type 2 classical cadherins (Suzuki, 1996). The type 1 cadherins include the three originally identified cadherins; E-cad, N-cad, and P-cad whereas human cad-6, -7, -8, -9, -10, -11, -12, -14, and -20 have been assigned to the type 2 cadherin subfamily (Nose and Takeichi, 1986; Nagafuchi *et al.*, 1987; Hatta *et al.*, 1988; Suzuki *et al.*, 1991; Tanihara *et al.*, 1994; Shibata *et al.*, 1997; Shimoyama *et al.*, 2000; Kools *et al.*, 2000).

The type 1 and type 2 classical cadherins share common structural features (Suzuki *et al.*, 1991; Tanihara *et al.*, 1994). Although the over-all amino acid sequence homology between these two subfamilies is low, the cytoplasmic domains of the type 1 and type 2 cadherins are highly conserved (Suzuki *et al.*, 1991). These domains interact with a family of proteins, known as the catenins (Aberle *et al.*, 1996). β-catenin and γ-catenin interact with the cadherins in a mutually exclusive manner (Butz and Kemler, 1994; Nathke *et al.*, 1994). α-catenin, in turn, links both cadherin-catenin complexes to the actin-based cytoskeleton by direct interaction (Rimm *et al.*, 1995a) or via α-actinin (Knudsen *et al.*, 1995). $p120^{ctn}$, a substrate for the *src* tyrosine kinases, has also been shown to interact with the cadherin cytoplasmic domain (Reynolds *et al.*, 1989; Reynolds *et al.*, 1992; Ohkubo and Ozawa, 1999). As $p120^{ctn}$ does not bind to α-catenin, this cytoplasmic protein is not believed to link the cadherins to the cytoskeleton (Daniel and Reynolds, 1995).

The cadherins are key morphoregulators (Takeichi, 1991; 1995). The spatiotemporal expression of both the type 1and type 2 classical cadherins is tightly regulated during embryonic development. For example, the differential expression of E-cad and cad-11 has been associated with the formation of mesodermal cell layers in the rodent embryo (Hoffmann and Balling, 1995; Simonneau *et al.*, 1995). The regulated expression of the type 1 cadherins has also been shown to govern the developmental fate of cells. In particular, the transfection of a full-length E-cad cDNA into embryonic stem cells lacking endogenous cadherin expression results in epithelial cell differentiation whereas N-cad expression promotes the formation of cartilage and neuroepithelial cell structures (Larue *et al.*, 1996). To date, the ability of the type 2 cadherins to modulate cellular differentiation remains poorly characterized.

Villous cytotrophoblasts of the human placenta undergo a process of cellular differentiation and fusion to form the syncytial trophoblast, a multinucleated cell that contributes to the majority of placental transport, immunoregulatory, and endocrine functions during pregnancy (Richart, 1961; Pierce and Midgley, 1963; Kliman *et al.*, 1986). We have recently determined that E-cad and cad-11 are differentially expressed during the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta (MacCalman *et al.*, 1996a). In particular, E-cad expression levels are high in freshly isolated cytotrophoblasts and decrease as the cells undergo aggregation and fusion to form multinucleated syncytium with time in culture. The loss of E-cad in these cultures is associated with a decrease in β-catenin expression and a concomitant increase in the expression levels of cad-11 (Chapter 2.2; Getsios *et al.*, 2000). In contrast, E-cad and β-catenin, but not cad-11, are readily detectable in non-fusing JEG-3 choriocarcinoma cells. Collectively, these observations have led us to hypothesize that cad-11 mediates the formation of multinucleated syncytium from mononucleate trophoblastic cells *in vitro*.

Here we report that the transfection of a mammalian expression vector containing a full-length cad-11 cDNA into mononucleate JEG-3 cells was capable of promoting the morphological and functional differentiation of these trophoblastic cells. In addition, antisense oligonucleotides, specific for cad-11, inhibited the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta. These studies not only add to our understanding of the adhesive mechanisms operative during trophoblast differentiation but provide useful insight into the cell biology of the type 2 cadherins.

## Materials and Methods

### *Tissues*

Term placentae (n=6) were obtained from women undergoing cesarean section. Follicular aspirates were collected from the ovaries of patients undergoing *in vitro* fertilization and embryo transfer. Research using human tissues was approved by the Committee for Ethical Review of Research Involving Human Subjects, University of British Columbia. All subjects provided informed consent for these studies.

### *Cell isolation and culture*

Granulosa-lutein cells were isolated from human ovarian follicles according to the methods described by Golos and Strauss (1987). Briefly, the follicular aspirates were centrifuged on a Ficoll-Paque gradient (Pharmacia Biotech Inc., Baie D'Urfe, PQ) at 1, 000 x g for 10 min at RT. The granulosa-lutein cells were collected from the gradient interface before being washed, resuspended, and plated in DMEM (Gibco BRL, Burlington, ON) containing 25 mM glucose, 25

mM HEPES, and 50 μg/ml gentamicin and supplemented with 10% heat-inactivated FCS (Hyclone Labs Inc., Logan, UT).

Villous cytotrophoblasts were isolated from human term placental tissues as previously described by Kliman *et al.* (1986). This method, which utilizes serial trypsin-DNase digestions, yields a highly enriched preparation of mononucleate cytotrophoblasts. Following isolation, the cells were resuspended and plated in the culture medium described above.

JEG-3 choriocarcinoma (American Type Culture Collection, Rockville, MD) and COS-7 cells (gift from CB Verchere, University of British Columbia, Vancouver, BC) were maintained in the culture medium described above and harvested from on-going cultures with 0.125 % trypsin in EDTA buffer.

*Expression vectors*

A full-length cad-11 cDNA (gift from ICOS Corp., Bothell, WA) was subcloned into the mammalian expression vector, pCMV/SPORT1 (Gibco BRL), using standard molecular biology techniques. The orientation and reading frame of two clones containing the cad-11 cDNA in the forward and reverse orientation (pCAD-11 and pRCAD-11, respectively) was confirmed by DNA sequence analysis. A pCMV/SPORT1 expression vector containing the β-galactosidase gene (p*lac*Z; Gibco BRL) was used to determine the transfection efficiency of pCAD-11 and pRCAD-11 in these studies.

To determine whether pCAD-11 was capable of directing the production of a mature cad-11 protein species that could be expressed on the cell surface, COS-7 cells were transfected with pCAD-11, pRCAD-11, or p*lac*Z (1.0 μg/ml) using the Fugene 6 transfection reagent (Roche Diagnostics, Laval, PQ) and cultured for a further 24 h.

The ability of exogenous cad-11 expression to modulate the morphological and functional differentiation of JEG-3 choriocarcinoma cells was then examined. JEG-3 cells (2.5 x $10^5$ cells) were seeded on glass coverslips (2 x 2 cm) and cultured for 12 h. The cells were then transfected with pCAD-11, pRCAD-11, or p*lac*Z (1.0 μg/ml) and cultured for a further 0, 12, 24, or 36 h.

*Antisense oligonucleotides*

Oligonucleotide sequences (18mers, 50% AT/GC content) were selected from the full-length human cad-11 cDNA (GenBank accession number L34056.1) using the Primer3 program (Whitehead Institute, Cambridge, MA). DNA sequences located near the 5' end of the cad-11 cDNA were compared to the human sequence databases of EMBL and GenBank. Two sequences were identified (5'-GGCGGCTTGTAAACAGTA-3' and 5'-CACGAAGAACTGGTTCCA-3'), corresponding to bp 168-185 and bp 324-341 of the cad-11 cDNA, respectively. Phosphothiorate-labeled antisense oligonucleotides (OB-1 and OB-2) complementary to these DNA sequences and the corresponding sense oligonucleotides (OB-3 and OB-4) were prepared (Nucleic Acid and Protein Synthesis Biotechnology Lab, University of British Columbia, Vancouver, BC) and used in these studies.

To determine whether OB-1 and OB-2 were capable of decreasing cad-11 mRNA levels in a specific manner, granulosa-lutein cells, which express cad-6, cad-11, and N-cad (MacCalman *et al.*, 1997a), were cultured in the presence of these antisense oligonucleotides. Granulosa-lutein cells (4 x $10^6$ cells) were seeded in 60 mm culture dishes and cultured in the presence of increasing concentrations (0, 1 or 5 μM) of antisense (OB-1 or OB-2) or sense oligonucleotides (OB-3 or OB-4) for a further 24 h. The concentrations of oligonucleotides used in these experiments were selected on the basis of previous studies (Cannigia *et al.*, 1997).

The ability of these antisense oligonucleotides to inhibit the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta was then examined. Mononucleate cytotrophoblasts (5 x $10^5$ cells) were seeded on glass coverslips (2 x 2 cm) and cultured in the presence of OB-1 or OB-2 (5 μM) for 24, 48, or 72 h. Cytotrophoblasts cultured in the presence of the sense oligonucleotides, OB-3 or OB-4 (5 μM), served as a control for these studies.

*Northern blot analysis*

Total RNA was prepared from cultures of granulosa-lutein cells or JEG-3 cells using the phenol-chloroform method of Chomczynski and Sacchi (1987). The RNA species were resolved by electrophoresis in 1% agarose gels containing 3.7% formaldehyde. Approximately 20 μg of total RNA were loaded in each lane. The fractionated RNA species were then transferred onto charged nylon membranes (Amersham Canada Ltd., Oakville, ON).

The Northern blots were probed with radiolabeled cDNAs specific for human cad-11 (MacCalman *et al.*, 1996a), N-cad (MacCalman *et al.*, 1997a), cad-6 (Getsios *et al.*, 1998a), βhCG (Coutifaris *et al.*, 1991), and a radiolabeled synthetic oligonucleotide specific for 18S rRNA (Szyf *et al.*, 1990) as previously described (MacCalman *et al.*, 1992).

*Antibodies*

Two mouse monoclonal antibodies specific for human cad-11 (113E and 113H) were used in these studies (gift from ICOS Corp., Bothell, WA). Mouse monoclonal antibodies directed against human E-cad and β-catenin were purchased from Transduction Labs (Lexington, KY). A mouse monoclonal antibody directed against 5-bromo-2'-deoxy-uridine (BrdU) was

purchased from Roche Diagnostics. Non-specific isotype-matched mouse monoclonal antibodies were purchased from Dako Corp. (Carpenteria, CA) and used as negative controls in these studies.

*Western blot analysis*

COS-7 cells or villous cytotrophoblasts were washed three times in PBS and incubated in 100 µl of cell lysis buffer (10 mM Tris-HCl, pH 7.5 containing 0.5% NP-40, 0.5 mM $CaCl_2$, and 1.0 mM PMSF) at 4 °C for 30 min on a rocking platform. The cell lysates were centrifuged at 10,000 x g for 20 min and the supernatants used for Western blot analysis. The concentration of protein in the cell lysates was determined using the BCA kit (Pierce Chemicals, Rockford, IL). Western blots containing aliquots (20 µg) of the cell lysates were prepared and immunoblotted as previously described (MacCalman *et al.*, 1996a). The Amersham ECL system was used to detect antibody bound to antigen.

*Immunocytochemistry*

JEG-3 cells were fixed in 4% paraformaldehyde for 15 min at RT. Immunocytochemistry was performed according to the methods of Cartun and Pedersen (1989) and included sequential incubations in 10% normal horse serum, primary antiserum at 37 °C for 1 h, secondary biotinylated antibody at 37 °C for 45 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37 °C for 30 min, and three washes (5 min each) in PBS. The cells were then exposed to chromagen reaction solution (0.035% diaminobenzidine and 0.03% $H_2O_2$) for 10 min, washed in tap water for 5 min, counterstained in haematoxylin, dehydrated, cleared, and mounted.

*Indirect immunoflourescence*

Villous cytotrophoblasts were fixed in 100% methanol for 5 min at –20 °C. The fixed cells were washed in PBS and non-specific reactions blocked by incubation with 10% normal horse serum. The coverslips were then exposed to primary antiserum for 1 h at 37 °C. After washing with PBS, the cells were incubated with a fluorescein-labeled anti-mouse antibody for 30 min at 37 °C. The coverslips were mounted on glass slides using 80% glycerol and visualized using an Axioplan microscope equipped with epifluorescence (Carl Zeiss, Toronto, ON).

*BrdU incorporation*

A BrdU labeling and detection kit (BrdU labeling and detection kit II; Roche Diagnostics) was used to determine the effects of ectopic cad-11 expression on JEG-3 cell proliferation. Briefly, JEG-3 cells were cultured in the presence of BrdU (10 μM) prior to being fixed in 70% ethanol containing 15 mM glycine, pH 2.0 for 20 min at –20 °C. BrdU was immunolocalized in these fixed cell cultures using a mouse monoclonal antibody directed against BrdU.

**Results**

*pCAD-11 directs the expression of a mature cad-11 protein species in COS-7 cells*

Western blot analysis, using a mouse monoclonal antibody directed against human cad-11, revealed a single 125 kDa protein species in extracts prepared from COS-7 cells transfected

with pCAD-11 (Fig. 3.2.1). In contrast, we failed to detect this cad-11 protein species in the cultures of COS-7 cells transfected with pRCAD-11 or p*lac*Z.

*pCAD-11 promotes the terminal differentiation and fusion of JEG-3 cells*

β-galactosidase activity in JEG-3 cells transfected with p*lac*Z demonstrated that >70% of the cells had been transfected (Appendix II).

The transfection of pCAD-11 into JEG-3 cells also correlated with distinct morphological changes in these cultures. In particular, large multinucleated syncytial structures were first detected in JEG-3 cells transfected with pCAD-11 for 24 h. These cellular structures were predominant in these cultures at 36 h. In contrast, multinucleated syncytium was not observed in JEG-3 cells transfected with pRCAD-11 or p*lac*Z at any of the time points examined in these studies.

E-cad and β-catenin were immunolocalized to areas of cell-cell contact in JEG-3 cells transfected with pRCAD-11 or p*lac*Z at all of the time points examined in these studies (Figs. 3.2.2 and 3, respectively). A marked reduction in the intensity of E-cad and β-catenin immunostaining was observed in cultures of JEG-3 cells transfected with pCAD-11 at 24 h. Furthermore, we failed to detect E-cad and β-catenin immunostaining in these cultures after 36 h. In contrast, cad-11 immunostaining was not observed in JEG-3 cells transfected with pRCAD-11 or p*lac*Z at any of the time points examined in these studies but was readily detectable in cultures transfected with pCAD-11 for 24 and 36 h (Fig. 3.2.4).

There was a marked reduction in the level of BrdU incorporation in JEG-3 cells transfected with pCAD-11 for 24 h (Fig. 3.2.5). In contrast, the transfection of pRCAD-11 or p*lac*Z did not have a marked effect on DNA synthesis in these cell cultures.

A single βhCG mRNA transcript (1.1 kb) was detected in all of the total RNA extracts prepared from the JEG-3 cell cultures (Fig. 3.2.6). There was a marked increase in the levels of the βhCG mRNA transcript present in JEG-3 cells transfected with pCAD-11 for 36 h. In contrast, the levels of the mRNA transcript encoding this biochemical marker of trophoblast differentiation remained relatively constant following the transfection of pRCAD-11 or p*lacZ* into these cells.

*OB-1 and OB-2 decrease cad-11 mRNA levels in human granulosa-lutein cell cultures*

A single cad-11 mRNA transcript (4.4 kb) was detected in all of the total RNA extracts prepared from the isolated granulosa-lutein cells (Fig. 3.2.7). OB-1 decreased cad-11 mRNA levels in these primary cell cultures at all of the concentrations examined in these studies. The most marked decrease in the levels of the mRNA transcript encoding cad-11 was observed in cells cultured in the presence of OB-1 (5 μM). In contrast, the steady-state levels of the two major N-cad mRNA species (4.3 kb and 4.0 kb) and the single cad-6 mRNA transcript (4.1 kb) remained relatively constant in granulosa-lutein cells cultured in the presence of this antisense oligonucleotide. The addition of the sense oligonucleotide, OB-3, to the culture medium did not have a marked effect on the levels of the mRNA transcripts encoding the three cadherin subtypes present in human granulosa-lutein cells at any of the concentrations examined in these studies. Similar results were obtained using granulosa-lutein cells cultured in the presence of OB-2 or OB-4.

*OB-1 inhibits the terminal differentiation and fusion of villous cytotrophoblasts isolated from human term placentae*

Western blot analysis demonstrated that villous cytotrophoblasts cultured in the presence of the antisense oligonucleotide, OB-1 (5 μM), failed to up-regulate cad-11 expression levels at any of the time points examined in these studies (Fig. 3.2.8). Instead, the levels of E-cad expression remained relatively constant in these primary cell cultures until the termination of these studies at 72 h. There was, however, a marked reduction in E-cad expression in villous cytotrophoblasts cultured in the presence of the sense oligonucleotide, OB-3 (5 μM), for 48 h. A further decrease in E-cad expression was observed in these primary cell cultures at 72 h. In contrast, cad-11 expression continued to increase in the trophoblastic cells cultured in the presence of OB-3 until the termination of these studies at 72 h.

Villous cytotrophoblasts cultured in the presence of OB-1 were capable of forming large cellular aggregates after 72 h of culture, as determined by phase contrast light microscopy (Fig. 3.2.8). E-cad expression was subsequently immunolocalized to areas of cell-cell contact in these primary cell cultures, demonstrating that these cellular aggregates were comprised of mononucleate cytotrophoblasts. In contrast, we failed to detect E-cad immunostaining in the multinucleated syncytium that formed in the trophoblastic cells cultured in the presence of OB-3 for 72 h.

**Discussion**

E-cad and cad-11 appear to have distinct roles in the aggregation, terminal differentiation, and fusion of villous cytotrophoblasts isolated from human term placentae. For example, although E-cad has been localized to mononucleate trophoblastic cells, function-perturbing antibodies generated against this CAM are capable of inhibiting the formation of syncytium in these cultures of villous cytotrophoblasts (Coutifaris *et al.*, 1991). However, these morphological effects are believed to be due to the inability of the mononucleate cytotrophoblasts to undergo

aggregation prior to the onset of terminal differentiation and fusion. Further evidence that E-cad expression is not sufficient to promote the formation of multinucleated syncytium in these primary cell cultures has been obtained from studies examining the effects of tyrosine phosphorylation on trophoblast differentiation (Rebut-Bonneton *et al.*, 1993). Villous cytotrophoblasts cultured in the presence of herbimycin A, an inhibitor of tyrosine kinase activity (Murikama *et al.*, 1988), were capable of up-regulating E-cad expression levels but failed to undergo terminal differentiation and fusion (Rebut-Bonneton *et al.*, 1993). Here, we have demonstrated that E-cad expression levels are maintained in villous cytotrophoblasts cultured in the presence of antisense oligonucleotides specific for cad-11. These cells were capable of forming large cellular aggregates but did not undergo terminal differentiation and fusion to form syncytium with time in culture. Collectively, these observations suggest that E-cad mediates the aggregation of mononucleate trophoblastic cells whereas cad-11 expression is required for the formation of multinucleated syncytium in these primary cell cultures.

The ectopic expression of cad-11 in JEG-3 choriocarcinoma cells resulted in the loss of E-cad expression from the surface of these cells. A switch from E-cad to cad-11 expression has also been observed during the terminal differentiation and fusion of isolated villous cytotrophoblasts (MacCalman *et al.*, 1996a). These observations suggest that an increase in cad-11 expression is capable of modulating the expression levels of endogenous E-cad in mononucleate trophoblastic cells. Recent studies have demonstrated cell-specific differences in the ability of exogenous cadherins to regulate the expression levels of endogenous cadherin subtypes. For example, the introduction of a full-length N-cad cDNA into oral squamous carcinoma cell lines resulted in a loss of E-cad expression and induced a fibroblastic phenotype in these cell cultures (Islam *et al.*, 1996). In contrast, ectopic N-cad expression was shown to increase the motility and invasive capacity of breast cancer cell lines without altering the levels of E-cad expression (Nieman *et al.*, 1999a). Furthermore, the introduction of a full-length cad-5

cDNA into cultured endothelial cells was capable of modulating junctional complex formation by altering the localization but not the expression levels of the endogenous cadherin subtypes present in these cells (Navarro *et al.*, 1998). To date, the cellular mechanisms that regulate the coordinate expression of transfected full-length cadherin cDNAs with the endogenous cadherin subtypes present in these different cell types remain poorly defined.

The ectopic expression of both type 1 and type 2 classical cadherins has been shown to increase β-catenin expression levels in several cell lines, including murine fibroblastic L cells, baby hamster kidney cells and human epidermoid carcinoma cells (Redfield *et al.*, 1998; Nieman *et al.*, 1999; Shimoyama *et al.*, 2000). This increase is believed to result from the stabilization of β-catenin when it is complexed to the exogenous cadherin subtype (Papkoff, 1997). In contrast, the transfection of a full-length cad-11 cDNA into JEG-3 cells was associated with a loss of β-catenin from the surface of these cells. The expression patterns of E-cad and β-catenin in these transfected JEG-3 cell cultures correlate with those previously observed in villous cytotrophoblasts undergoing terminal differentiation and fusion *in vitro* (Chapter 2.2; Getsios *et al.*, 2000). As β-catenin was shown to interact with E-cad, but not cad-11, in these primary cell cultures, it would seem likely that the overexpression of cad-11 is not capable of stabilizing this cytoplasmic protein in JEG-3 cells. In view of these observations, it is tempting to speculate that cad-11 mediates the disassembly and/or down-regulation of E-cad/β-catenin complexes, resulting in the morphological differentiation of mononucleate trophoblastic cells *in vitro*.

The terminal differentiation and fusion of villous cytotrophoblasts is associated with a reduction in cellular proliferation and an increase in the synthesis and secretion of βhCG in these cells (Richart, 1961; Pierce and Midgley, 1963; Kliman *et al.*, 1986). Previous studies have indicated that the cellular mechanisms that regulate the biochemical differentiation of human trophoblastic cells are distinct from those involved in the formation of multinucleated syncytium

*in vitro* (Kao *et al.*, 1988; 1992). For example, the intracellular secondary messenger, cAMP, is capable of increasing βhCG mRNA levels in JEG-3 choriocarcinoma cells (Burnside *et al.*, 1985) but does not promote the formation of multinucleated syncytium in these cultured cells (Coutifaris *et al.*, 1991; MacCalman *et al.*, 1996a). Similarly, the transfection of a full-length Cx-26 cDNA into JEG-3 cells resulted in a decrease in cellular proliferation and a concomitant increase in βhCG secretion with time in culture (Hellmann *et al.*, 1999). However, large multinucleated syncytial structures were not observed in these transfected cell cultures. In contrast, cad-11 was not only capable of promoting the morphological differentiation of JEG-3 cells but reduced cellular proliferation and increased the levels of the βhCG mRNA transcript present in these cultures.

In summary, we have determined that cad-11 mediates the morphological and functional differentiation of human cytotrophoblasts *in vitro*. To our knowledge, these studies are the first to define a role for this CAM during cellular differentiation. Although the cellular mechanism(s) by which cad-11 promotes the formation of multinucleated syncytium remain poorly understood, our findings suggest that this developmental process involves the coordinated down-regulation of E-cad and β-catenin expression levels in mononucleate trophoblastic cells.

Fig. 3.2.1: Autoradiogram of a Western blot containing total protein extracted from COS-7 cells transfected with p*lac*Z, pRCAD-11, or pCAD-11 for 24 h (lanes a-c, respectively). Western blot analysis was performed using a mouse monoclonal antibody directed against human cad-11 (113H). The Amersham ECL system was used to detect antibody bound to antigen. The relative electrophoretic mobilities of the molecular weight markers are shown on the left hand side of the immunoblot.

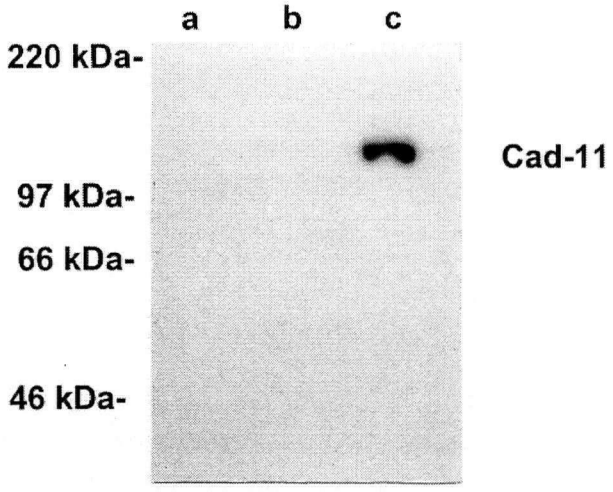

a
b
c
220 kDa-
97 kDa-
66 kDa-
46 kDa-
Cad-11

Fig. 3.2.2: Photomicrographs of E-cad expression in JEG-3 cells transfected with pCAD-11 and cultured for 12, 24, or 36 h (panels A-C, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against human E-cad. A negative control in which JEG-3 cells were transfected with pRCAD-11 for 36 h is shown in panel D. The scale bars represents 20 μm.

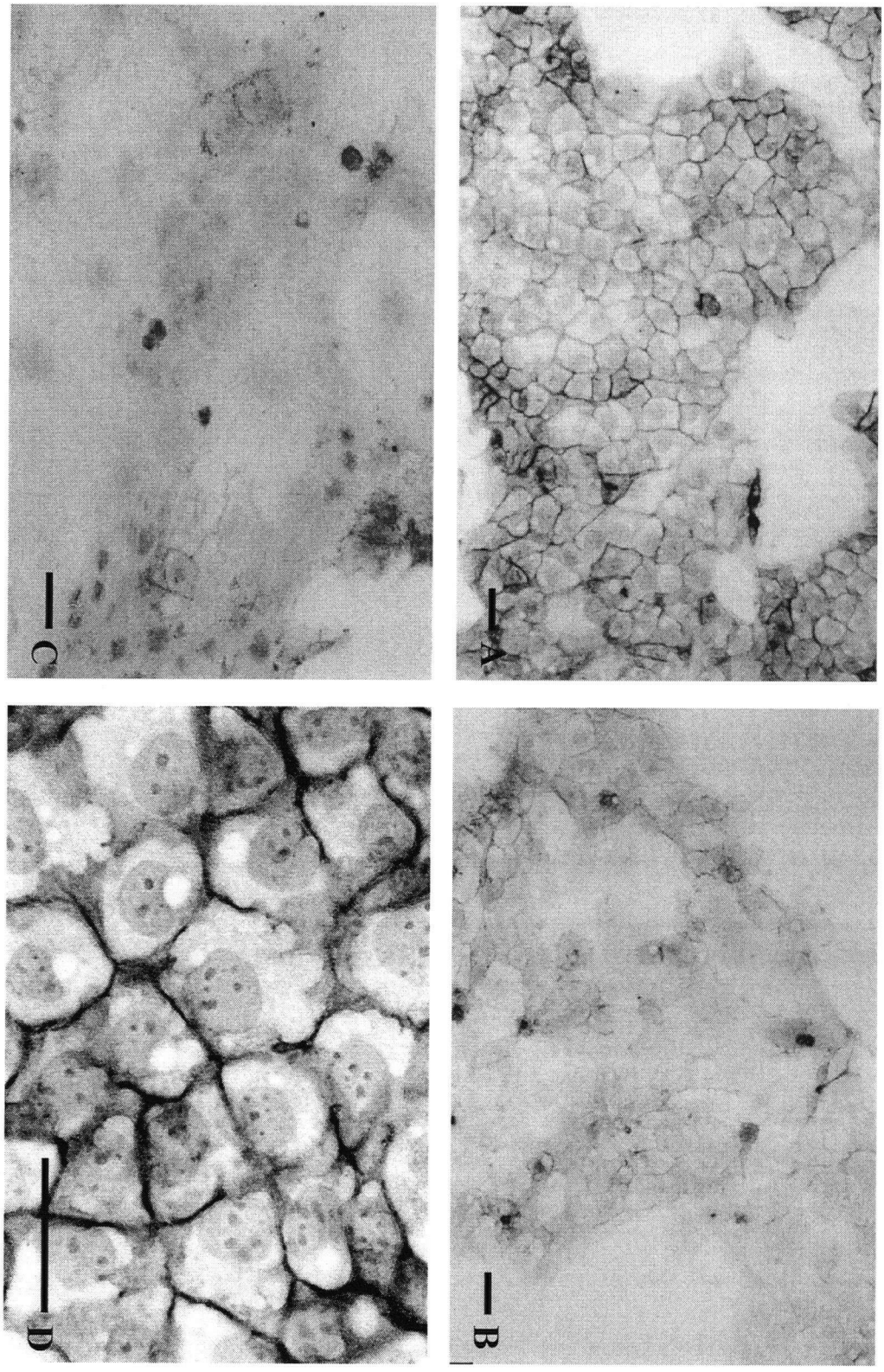

C
A
D
B

Fig. 3.2.3: Photomicrographs of β-catenin expression in JEG-3 cells transfected with pCAD-11 and cultured for 12, 24, or 36 h (panels A-C, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against human β-catenin. A negative control in which JEG-3 cells were transfected with pRCAD-11 for 36 h is shown in panel D. The scale bars represents 20 μm.

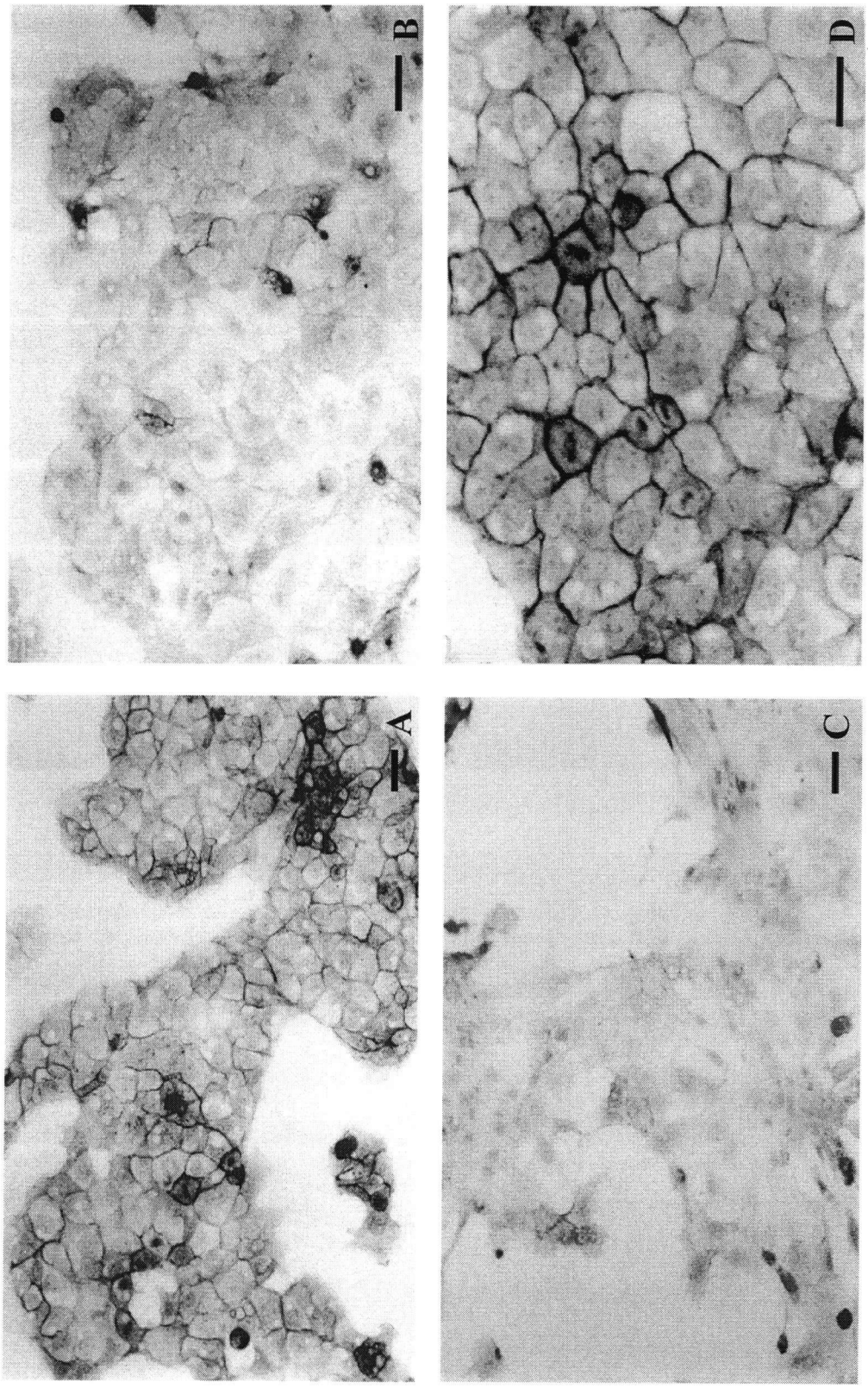

B
D
A
C

Fig. 3.2.4: Photomicrographs of cad-11 expression in JEG-3 cells transfected with pCAD-11 and cultured for 24 or 36 h (panels B and C, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against human cad-11 (113E). A negative control in which JEG-3 cells were transfected with pRCAD-11 for 36 h is shown in panel A. The scale bar represents 20 μm.

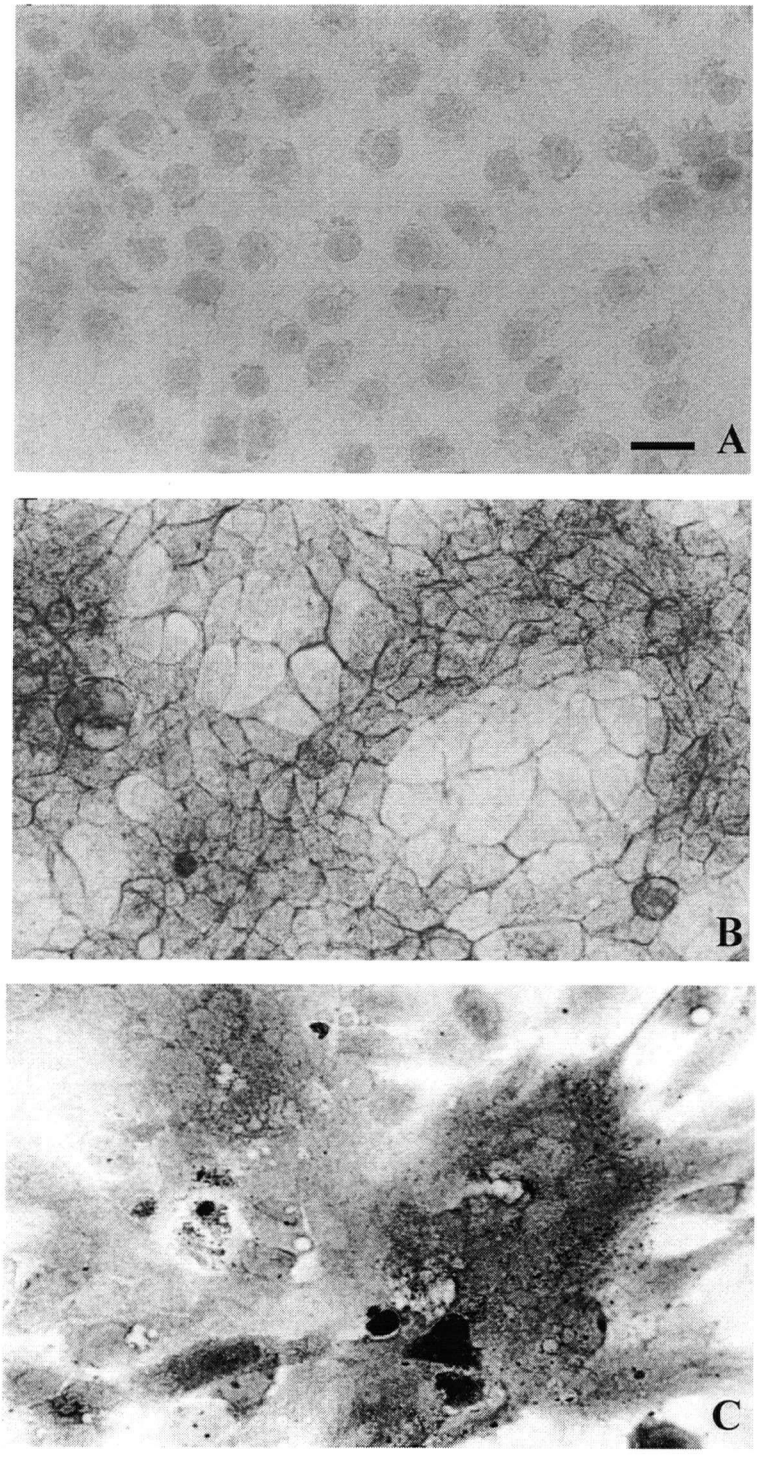

A
B
C

Fig. 3.2.5: Photomicrographs of BrdU incorporation in JEG-3 cells transfected with p*lacZ*, pRCAD-11, or pCAD-11 for 24 h (Panels A-C, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against BrdU. The scale bar represents 40 μm.

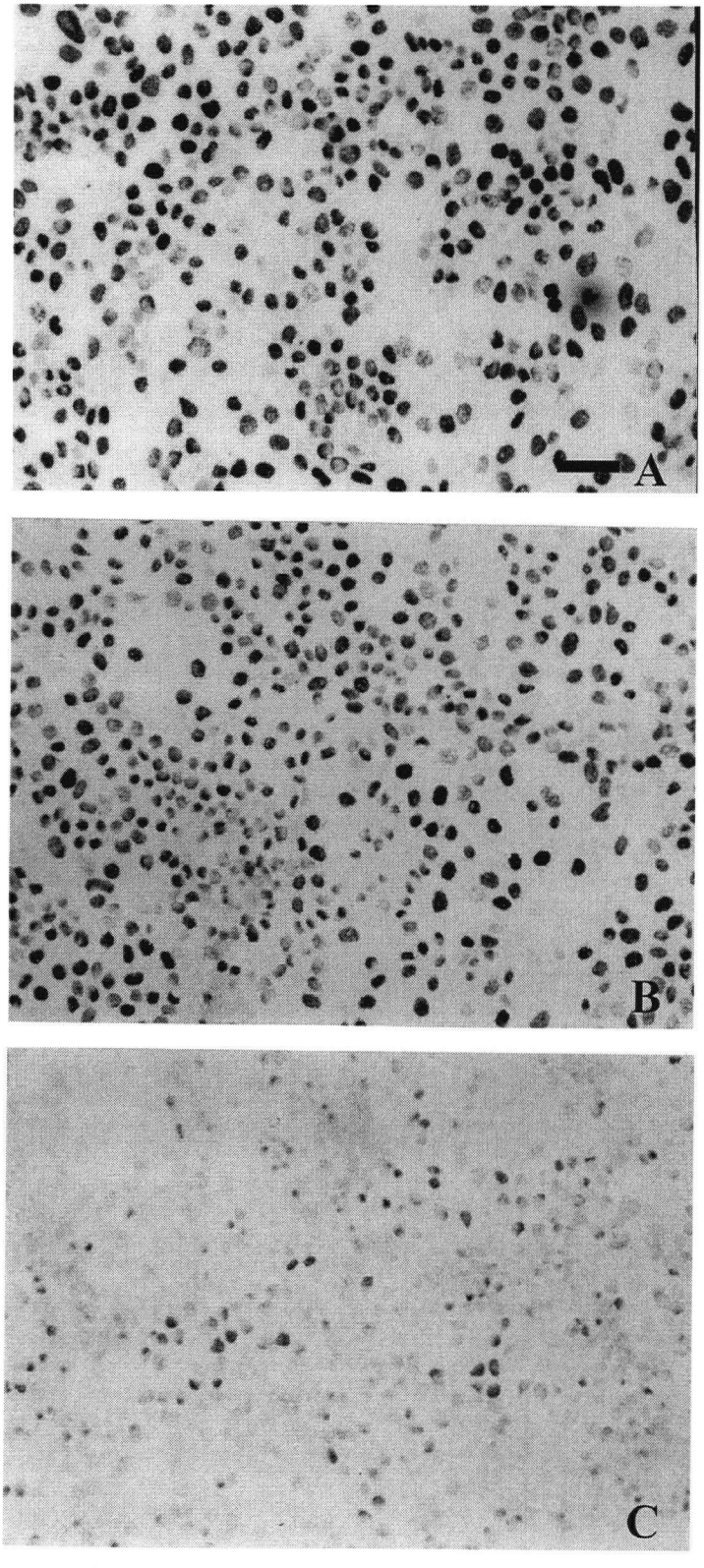

A
B
C

Fig. 3.2.6: Autoradiograms of a Northern blot containing total RNA extracted from JEG-3 cells transfected with p*lac*Z, pRCAD-11, or pCAD-11 and cultured for 0 (lane a) or 36 h (lanes b-d, respectively). This blot was probed for βhCG (upper panel) or 18S rRNA (lower panel). The positions of the 28S and 18S rRNA are shown on the left hand side of the panel.

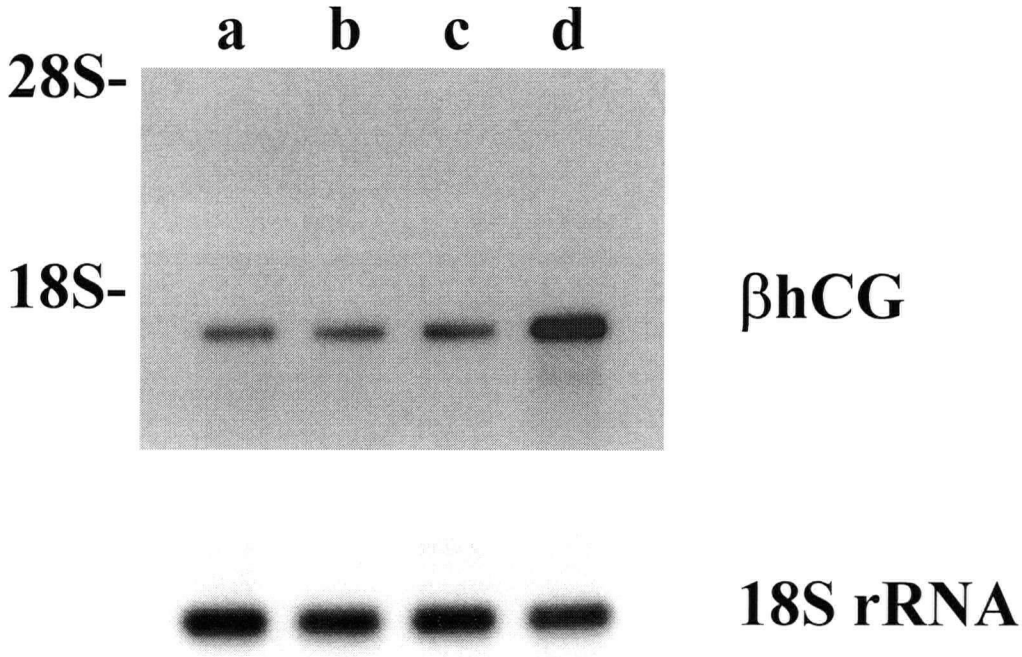

a
b
c
d
28S-
18S-
βhCG
18S rRNA

Fig. 3.2.7: Autoradiograms of a Northern blot containing total RNA extracted from human granulosa-lutein cells cultured in the presence of 0, 1, or 5 μM of OB-1 (lanes a, b, and d, respectively) or the corresponding sense oligonucleotide, OB-3 (lanes c and e, respectively), for 24 h. This blot was probed for cad-11, N-cad, cad-6, or 18S rRNA. The positions of the 28S and 18S rRNA are shown on the left hand side of the panels.

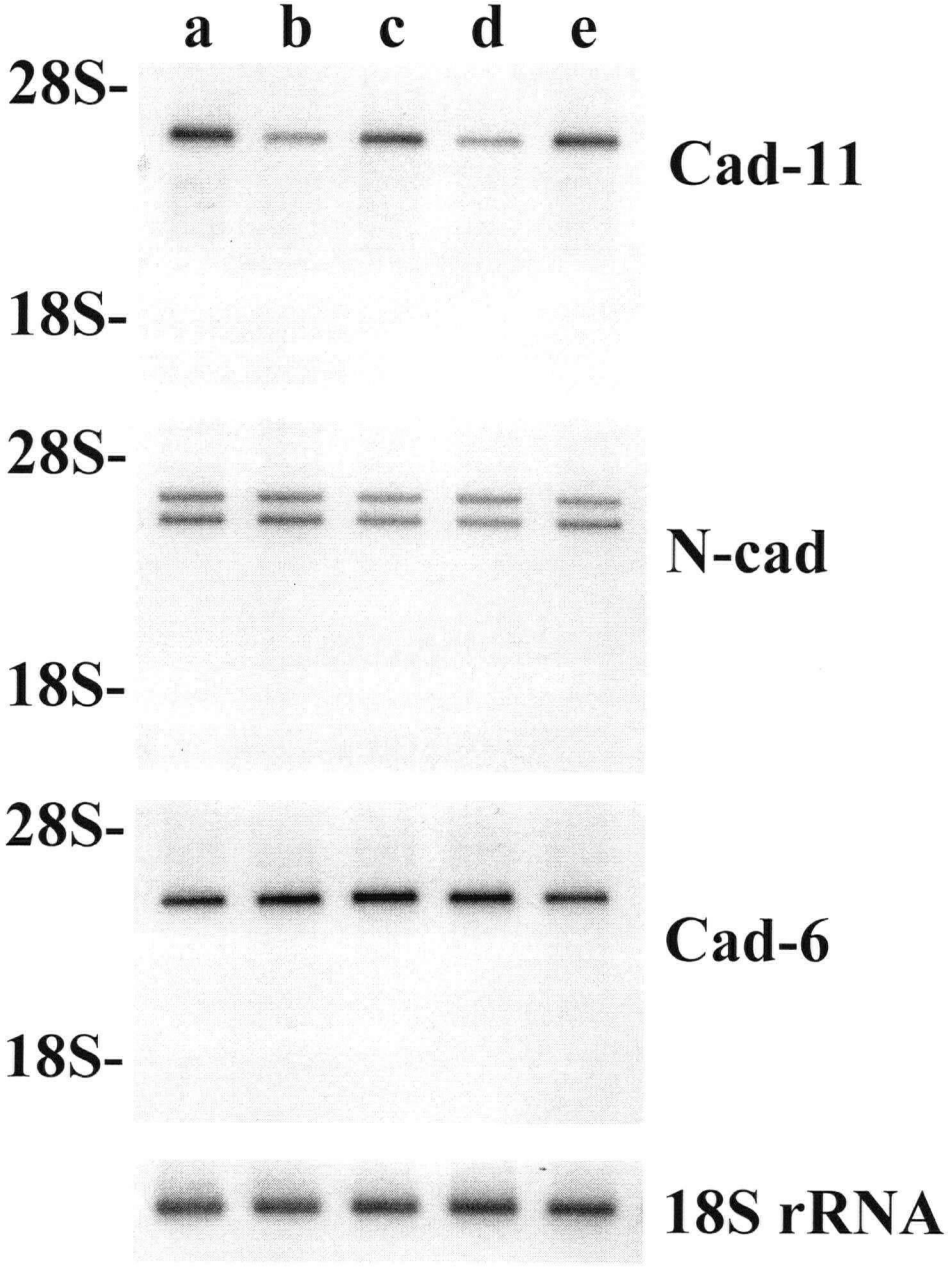
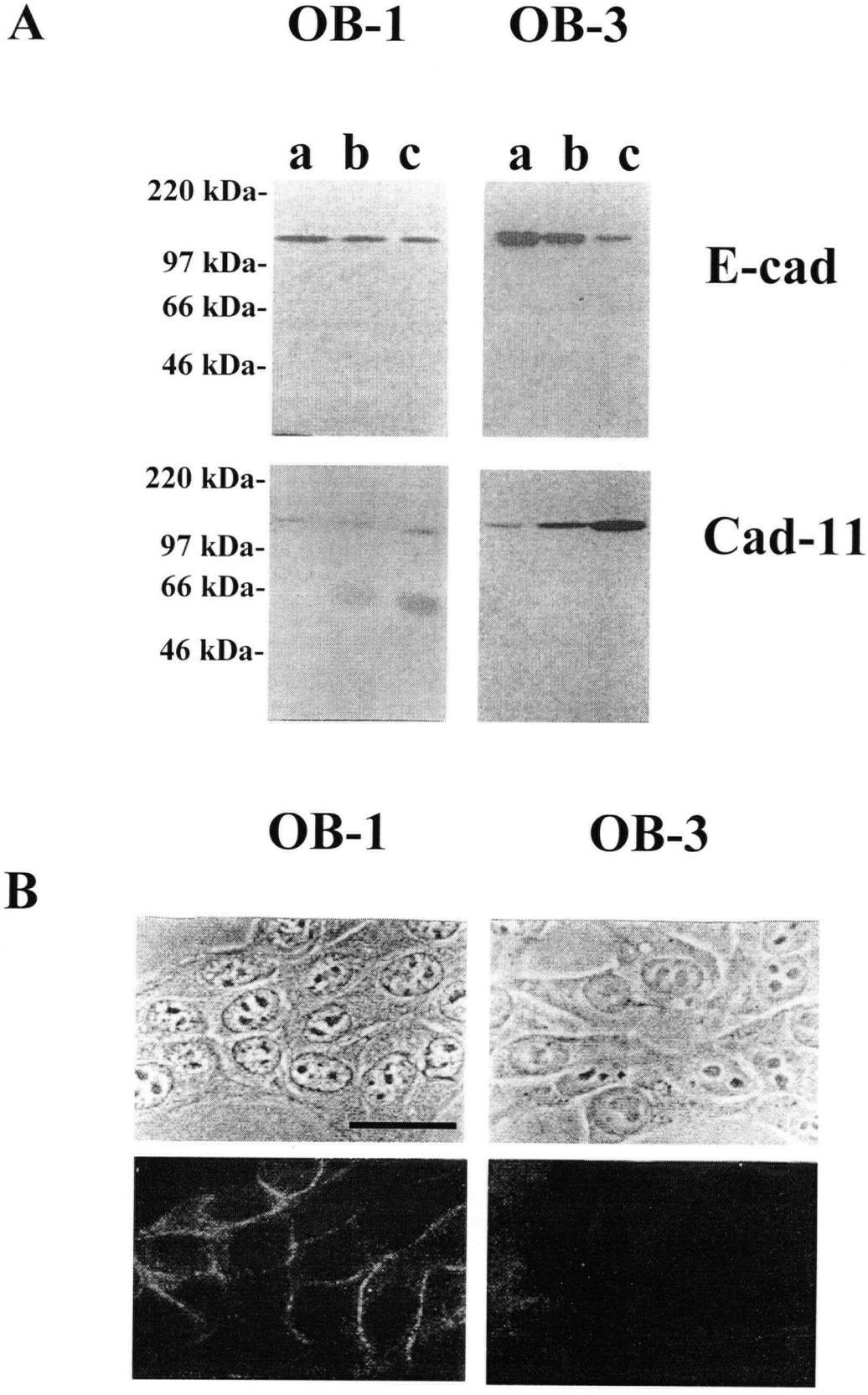

a b c d e
28S-
Cad-11
18S-
28S-
N-cad
18S-
28S-
Cad-6
18S-
18S rRNA

Fig. 3.2.8: (A) Autoradiograms of Western blots containing total protein extracted from villous cytotrophoblasts cultured in the presence of either OB-1 or OB-3 (5 μM) for 24, 48, or 72 h (lanes a-c, respectively). Western blot analysis was performed using mouse monoclonal antibodies directed against human E-cad or cad-11 (113H). The Amersham ECL system was used to detect antibody bound to antigen. The relative electrophoretic mobilities of the molecular weight markers are shown on the left hand side of the immunoblots.

(B) Photomicrographs of phase contrast light microscopy (upper panels) and the corresponding E-cad immunofluorescence (bottom panels) in villous cytotrophoblasts cultured in the presence of either OB-1 or OB-3 (5 μM) for 72 h. The cytotrophoblasts were fixed and immunostained with a mouse monoclonal antibody directed against human E-cad. The scale bar represents 20 μm.


A
OB-1
OB-3
a b c
a b c
220 kDa-
97 kDa-
66 kDa-
46 kDa-
E-cad
220 kDa-
97 kDa-
66 kDa-
46 kDa-
Cad-11
B
OB-1
OB-3

# CHAPTER IV: GENERAL DISCUSSION, SUMMARY, AND CONCLUSIONS

## 4.1: General Discussion

Membrane fusion processes play an important role both intracellularly, as is observed in the vesicular transport system that mediates the synthesis, sorting, and secretion of proteins (Rothman, 1996; Rothman and Wieland, 1996), and at the cell surface, as is observed in virus-cell and cell-cell fusion events (Hernandez *et al.*, 1996; Shemer and Podbilewicz, 2000). Cell-cell fusion is critical for embryonic development, mediating the fertilization of the egg and sperm (Wilson and Snell, 1998), the formation of myotubes from mononucleate myoblasts during skeletal muscle development (Nadal-Ginard, 1978), the formation of osteoclasts from bone marrow-derived mononuclear cells during bone remodeling (Roodman, 1996), and the formation of the syncytial trophoblast layer during human implantation and placentation (Kliman *et al.*, 1986). Cells must establish extensive adhesive interactions with one another prior to these cellular fusion processes, which results not only in alterations in cell morphology but also the physiology of these cells. For example, syncytial trophoblast formation is associated with changes in cellular proliferation, gene expression, and the endocrine properties of these trophoblastic cells (Kliman *et al.*, 1987; Ringler and Strauss, 1990; Morrish *et al.*, 1997). Previous studies have implicated the type 1 classical cadherin subtype, E-cad, in the aggregation of villous cytotrophoblasts isolated from the human term placenta (Coutifaris *et al.*, 1991). In these studies, we have demonstrated that the expression levels of E-cad, in conjunction with its associated proteins, α-, β-, γ-catenin, and $p120^{ctn}$, are down-regulated during this terminal differentiation process *in vitro* and *in vivo* (Chapter II). In contrast, an increase in cad-11 expression is capable of promoting the formation of multinucleated syncytium in mononucleate trophoblastic cell cultures (Chapter III).

The regulated expression of the classical cadherins and catenins has been previously correlated with the formation of multinucleated myotubes in skeletal muscle. For example, N-cad (Hahn and Covault, 1992; Mege *et al.*, 1992), cad-11 (Markus *et al.*, 1999), and cad-15 (Donalies *et al.*, 1991; Pouliot *et al.*, 1994; Rose *et al.*, 1994) have been identified in developing skeletal muscle and are expressed during myoblast differentiation *in vitro*. The expression levels of these three classical cadherin subtypes decrease as these cells undergo terminal differentiation and fusion to form multinucleated myotubes (Mege *et al.*, 1992; Pouliot *et al.*, 1994; Rose *et al.*, 1994; Markus *et al.*, 1999). As cad-11 expression is down-regulated during this developmental process, it is highly unlikely that this CAM mediates the formation of multinucleated myotubes *in vitro*. In addition, the expression of the cadherin/catenin complexes in these myoblast cultures decrease during this terminal differentiation process (Kuch *et al.*, 1997). Although the expression pattern of cad-4 during myoblast fusion *in vitro* has not been determined, this CAM is expressed in developing skeletal muscle and can promote the formation of skeletal muscle tissues structures when transiently expressed in murine embryonic stem cells (Rosenberg *et al.*, 1997). Collectively, these observations suggest that the adhesive mechanisms that mediate myoblast fusion are distinct from those operative during syncytial trophoblast formation in humans.

Classical cadherins have also been detected in multinucleated osteoclasts of human and murine bones (Mbalaviele *et al.*, 1995). In particular, E-cad expression increases during the aggregation of isolated murine bone marrow mononuclear cells and is subsequently down-regulated during the formation of multinucleated osteoclasts with time in culture. Inhibition of E-cad function, using function-perturbing antibodies or synthetic peptides containing the CAR sequence, HAV, was capable of reducing the number of multinucleated cells that formed in these primary cell cultures. This cellular differentiation and fusion process is also dependent on the heterotypic interactions that develop between mononuclear osteoclast precursor cells and the surrounding osteoblastic and stromal cells (Suda *et al.*, 1992). These cellular interactions are

mediated, at least in part, by the type 2 classical cadherin subtype, cad-6 (Mbalaviele *et al.*, 1998). The expression patterns of α-, β-, γ-catenin, and $p120^{ctn}$ were not determined in these studies. Similarly, cad-11 expression in these primary cell cultures has not been examined. As cad-11 is expressed in murine and human osteoblasts (Okazaki *et al.*, 1994; Cheng *et al.*, 1998; Lecanda *et al.*, 2000; Shin *et al.*, 2000), it is tempting to speculate that this CAM plays a role in osteoblast-osteoclast precursor cell interactions, thereby regulating the terminal differentiation and fusion of osteoclasts *in vitro*.

The cytoplasmic domains of E-cad and cad-11 are highly conserved in two regions; the membrane proximal $p120^{ctn}$ binding region and the carboxy terminal β/γ-catenin binding region (Nagafuchi *et al.*, 1987; Suzuki *et al.*, 1991; Tanihara *et al.*, 1994a; Berx *et al.*, 1995). In addition, cad-11 has been shown to interact with β-catenin in several human cancer lines, including those derived from the bone, breast, and stomach (Shibata *et al.*, 1996; Kashima *et al.*, 1999; Pishvaian *et al.*, 1999). In view of these observations, we examined the expression pattern(s) of the catenin subtypes, α-, β-, γ-catenin, and $p120^{ctn}$, during a cellular differentiation process that involved a switch in the expression of these two classical cadherin subtypes. We determined that the expression of α-, β-, γ-catenin, and $p120^{ctn}$ declined during the formation of multinucleated syncytium in a manner similar to E-cad, but not cad-11 expression. As the intervening amino acid sequences between the catenin binding regions of E-cad and cad-11 are less well-conserved than in these two regions, it is possible that cad-11 interacts with novel cytoplasmic proteins/catenin subtypes during the terminal differentiation and fusion of human villous cytotrophoblasts *in vitro*. Recent studies have demonstrated that several proteins that contain *arm* repeat domains, including δ-catenin, *arm* repeat protein deleted in velo cardio facial syndrome (ARVCF), and p100 are capable of interacting with the cytoplasmic domains of the classical cadherins (Staddon *et al.*, 1995; Lu *et al.*, 1999; Kaufmann *et al.*, 2000; Mariner *et al.*,

2000). Furthermore, the tyrosine kinase adaptor protein, Shc (Xu *et al.*, 1997; Xu and Carpenter, 1999), the non-receptor tyrosine kinase, Fer (Arregui *et al.*, 2000), and the cytoskeletal organizing protein, palladin (Parast and Otey, 2000), form complexes with the classical cadherin cytoplasmic domains in a catenin-independent manner. The ability of these cytoplasmic proteins to interact with cad-11 during the terminal differentiation and fusion of human trophoblastic cells remains to be determined.

The extracellular domain of cad-11 may also be involved in the formation of multinucleated syncytium in the human placenta. For example, cad-11 has been localized to the apical ruffled border of the syncytial trophoblast in sections of chorionic villi prepared from the human term placenta (MacCalman *et al.*, 1996a; 1997a). Actin-based microvillous structures are concentrated in this membrane domain of the human syncytial trophoblast layer *in vivo* (Boyd and Hamilton, 1970). In addition, microvilli are present at the sites of cytototrophoblast-cytotrophoblast fusion in primary cultures established from the human term placenta and are believed to play a role in cell-cell-fusion processes (Bax *et al.*, 1989; Nelson and Snell, 1998). As cad-11 was immunolocalized to the surface of transfected JEG-3 cell cultures prior to their morphological differentiation, it is tempting to speculate that this CAM mediates a late cellular adhesive event that is a prerequisite for fusion in human trophoblastic cells. Previous studies have used mutant cadherin cDNA constructs lacking either the extracellular or cytoplasmic domains of these CAMs in order to determine the relative contributions of these regions to type 1 classical cadherin function (Kintner, 1992; Dufour *et al.*, 1994; Levine *et al.*, 1994; Kuhl *et al.*, 1996; Zhu and Watt, 1996; Nieman *et al.*, 1999b; Troxell *et al.*, 1999). The generation of mutant cad-11 cDNA constructs is not likely to provide insight into trophoblast differentiation and fusion as the extracellular domain-deleted cDNA constructs described to date disrupt cadherin-mediated interactions by down-regulating endogenous cadherin expression levels and not by interfering with cadherin-mediated homophilic interactions (Zhu and Watt, 1996; Nieman *et al.*,

1999b; Troxell *et al.*, 1999). Chimeric cDNA constructs composed of the extracellular and cytoplasmic domains of cad-11 and E-cad, the endogenous cadherin subtype expressed by mononucleate JEG-3 cells, should provide a useful alternative and will be used in future studies to determine the cad-11 subdomains required to mediate fusion in cultured mononucleate trophoblastic cells.

It is unlikely that cad-11, alone, mediates membrane fusion during trophoblast differentiation since it does not share some essential structural elements of fusion proteins that have been identified in studies examining the process of viral entry into a host cell (Hernandez *et al.*, 1996; Shemer and Podbilewicz, 2000). For example, these viral fusion proteins are all type 1 integral membrane glycoproteins that contain a short (approximately 16-26 amino acids in length) membrane proximal peptide sequence that is relatively hydrophobic in nature and which mediates the fusion between the viral and host cell membrane (Durell *et al.*, 1997). This fusogenic amino acid sequence is not present in the extracellular domain of the full-length human cad-11 cDNA used in these studies. In addition, the transmembrane domain of viral envelope glyocoproteins is involved in the process of membrane fusion (Cleverly and Lenard, 1998), whereas this domain contributes to lateral interactions of the classical cadherins in the plasma membrane (Huber *et al.*, 1999). The induction of cell-cell adhesion results in conformational changes in fusion proteins that allows for their integration into the adjacent cell membrane (Blobel *et al.*, 1992). Moreover, the type 1 classical cadherins have been shown to interact with other integral membrane glycoproteins at the cell surface, including the protein tyrosine phosphatase1B-like phosphatase (PTP1B-LP; Balsamo *et al.*, 1996), the leukocyte-related protein PTP (LAR-PTP; Kypta *et al.*, 1996), and the member of the Ig gene superfamily of CAMs, nectin (Tachibana *et al.*, 2000). Collectively, these observations suggest that cad-11 may promote the terminal differentiation and fusion of mononucleate trophoblastic cells by interacting with other cell surface proteins. One such candidate binding partner is syncytin, a

recently identified envelope glycoprotein of the human endogenous retrovirus, HERV-W (Blond *et al.*, 1999). This integral membrane glycoprotein is expressed by the syncytial trophoblast of the human placenta, contains a putative fusion peptide sequence but not an adhesion-related domain, and is capable of promoting the terminal differentiation and fusion of BeWo choriocarcinoma cells (Blond *et al.*, 2000; Mi *et al.*, 2000). The ability of cad-11 to recruit syncytin to the site of intercellular fusion may explain, at least in part, how these two cellular proteins contribute to the morphological differentiation of human mononucleate trophoblastic cells *in vitro*.

Several studies have examined the ability of growth factors to regulate the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta (Morrish *et al.*, 1998). For example, EGF, which is produced by both the trophoblastic cells and decidual cells at the maternal-fetal interface (Hofmann *et al.*, 1991; 1992; Leach *et al.*, 1999), is capable of potentiating the formation of multinucleated syncytium in these primary cell cultures (Morrish *et al.*, 1987; 1997). Furthermore, a decrease in EGF-R activity has been shown to increase E-cad expression and reduce the number of multinucleated cells that form in these primary cell cultures (Rebut-Bonneton *et al.*, 1993). Coupled with the observation that the β-catenin is capable of linking E-cad with the EGF-R in human epidermoid carcinoma cell lines (Hoschuetzky *et al.*, 1994), it is likely that EGF modulates syncytial trophoblast formation by differentially regulating E-cad/catenin complexes and cad-11 in primary cultures of villous cytotrophoblasts isolated from the human term placenta. Although EGF has been shown to disrupt the association of E-cad with the actin-based cytoskeleton, this growth factor had no effect on the over-all expression levels of this CAM or α-, β-, γ-catenin and $p120^{ctn}$ in human breast cancer cell lines, suggesting that other growth factors may also be involved in regulating cadherin and catenin expression during trophoblast differentiation and fusion in vitro (Hazan and Norton, 1998).

We have previously determined that TGF-β1 is capable of increasing cad-11 mRNA levels and protein expression in extravillous cytotrophoblasts propagated from human first trimester placental explants (Appendix I). Cad-11 expression correlated with the aggregation and fusion of these primary cells cultured in the presence of TGF-β1. The effects of TGF-β1 on the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta are less clear. For example, Morrish *et al.* (1991) demonstrated a reduction in syncytial trophoblast formation and decreased βhCG secretion in villous cytotrophoblasts cultured in the presence of TGF-β1. Although Feinberg *et al.* (1994) confirmed the effects of TGF-β1 on βhCG secretion in these primary cell cultures, this growth factor was also capable of increasing the production of oncofetal Fn, a component of the ECM that was previously shown to increase during the terminal differentiation and fusion of these cells (Feinberg *et al.*, 1991). Furthermore, TGF-β1 has multiple effects on the expression of E-cad, β-, and γ-catenin in human colorectal, melanoma, and breast cancer cell lines (Miettenen *et al.*, 1994; Ilyas *et al.*, 1999; Janji *et al.*, 1999). Consequently, the ability of this growth factor to regulate E-cad, cad-11, α-, β-, γ-catenin, and $p120^{ctn}$ expression in human trophoblastic cells was not investigated in these studies.

Gonadal steroids are key regulators of E-cad and cad-11 expression in human reproductive tissues (MacCalman *et al.*, 1998). For example, 17β-estradiol (E2) is capable of increasing E-cad mRNA levels in the mouse ovary (MacCalman *et al.*, 1994a) and uterus (MacCalman *et al.*, 1994b) and in human breast carcinoma cell lines (Jednak *et al.*, 1993). The addition of progesterone (P4) but not E2 to the culture medium of isolated human endometrial stromal cells increased cad-11 mRNA levels and protein expression in a dose-dependent manner (Getsios *et al.*, 1998a). However, E2 was capable of potentiating the P4-mediated increase in cad-11 expression in these primary cell cultures (Chen *et al.*, 1998a). Finally, P4 was also shown to increase β-catenin mRNA levels in human endometrial stromal cells *in vitro* (Chen *et al.*,

1998b). Primary cultures of villous cytotrophoblasts produce P4 and aromatize exogenous androgens to estrogens *in vitro* (Kliman *et al.*, 1986; Ringler and Strauss, 1990). P4 receptors have also been detected in human villous cytotrophoblasts *in vitro* and *in vivo* (Shi *et al.*, 1993; Wang *et al.*, 1996; Rossmanith *et al.*, 1997), whereas the expression of E2 receptors in the trophoblastic cells of the human placenta has been disputed (Billiar *et al.*, 1997; Rossmanith *et al.*, 1997). The differences in E2 receptor expression profiles in the human placenta may be explained, at least in part, by the presence of distinct isoforms of these steroid hormone receptors (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). Recently, P4 has been shown to regulate the expression of the glycosylated phosphoprotein, osteopontin, in villous cytotrophoblasts isolated from the human term placenta (Omigbodun *et al.*, 1997). Similarly, E2 is capable of stimulating the morphological differentiation and increasing the production of βhCG in these primary cell cultures (Cronier *et al.*, 1999). In view of these observations, the ability of gonadal steroids to differentially regulate the expression of the classical cadherin and catenin subtypes in human trophoblastic cells seems warranted.

Our findings demonstrating a critical role for cad-11 in the terminal differentiation and fusion of human villous cytotrophoblasts could provide the basis for further studies examining the aberrant development of the placenta during pregnancy. Impaired trophoblastic cell fusion has been correlated with the development of pathological pregnancies (Benirschke and Kaufmann, 2000). For example, the histological examination of human placental specimens obtained from pregnancies complicated by intrauterine hypoxia has demonstrated an increase in the number of villous cytotrophoblasts and a decrease in the thickness of the syncytial trophoblast layer in these tissues (Fox, 1964). A reduction in the formation of multinucleated syncytial structures has also been observed in cultures of villous cytotrophoblasts isolated from the term placentae of patients diagnosed with preeclampsia (Pijnenborg *et al.*, 1996) and in villous cytotrophoblasts cultured under low oxygen tension, a condition that is believed to mimic

hypoxia *in vitro* (Alsat *et al.*, 1996; Jiang *et al.*, 2000). Previous studies have examined the expression of a variety of CAMs in the placentae of different pregnancy complications, but have focused primarily on the invasive subpopulation of extravillous cytotrophoblasts and the vasculature of chorionic villi. For example, Zhou *et al.* (1993; 1997b) have demonstrated alterations in the expression of the integrins, V-CAM-1, and PE-CAM in the extravillous cytotrophoblasts of preeclamptic placental bed biopsies. Similar differences in the levels of the mRNA transcripts that encode for PE-CAM in the placental vasculature of preeclamptic preganancies have not been reported (Lyall *et al.*, 1995). However, the expression of cad-5 is reduced in the chorionic villous endothelial cells present in placentae complicated by gestational diabetes (Babawale *et al.*, 2000). In addition, the subcellular distribution of cad-5 is altered in human umbilical vein endothelial cells (HUVEC) cultured in the presence of serum obtained from pre-eclamptic patients (Groten *et al.*, 2000). In our studies, we have demonstrated that a reduction in cad-11 expression inhibits the ability of isolated villous cytotrophoblasts to undergo terminal differentiation and fusion to form syncytium. Whether alterations in the regulated expression of cad-11 contribute to the morphological effects observed in the trophoblastic cells of these pregnancy complications will be the focus of future studies.

## 4.2: Summary and conclusions

These studies are the first to demonstrate a reduction in the expression of the catenin subtypes, α-, β-, γ-catenin, and $p120^{ctn}$ , during the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta. The expression pattern of these four catenins correlated with E-cad but not cad-11 expression in these primary cell cultures and in the mononucleate trophoblastic cells present in human chorionic villi. This is the first demonstration of the coordinated down-regulation of α-, β-, γ-catenin, and $p120^{ctn}$ expression during a cellular differentiation process that involves a switch in classical cadherin subtype expression. These observations suggest that cad-11 may interact with novel cytoplasmic proteins/catenin subtypes in the human syncytial trophoblast layer during human implantation and placentation. We also determined that cad-11 mediates the morphological and functional differentiation of human mononucleate trophoblastic cells *in vitro*. In particular, ectopic cad-11 expression promoted the terminal differentiation and fusion of mononucleate JEG-3 choriocarcinoma cells. Furthermore, a reduction in cad-11 expression was capable of inhibiting the formation of multinucleated syncytium in primary cultures of villous cytotrophoblasts. These observations provide the first demonstration of a biological role for this CAM in a mammalian cell. Collectively, these studies not only further our understanding of the cellular mechanisms that mediate the formation and organization of the human placenta but give us useful insight into the cell biology of the type 2 classical cadherin subfamily.

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# APPENDIX I: CORRELATION OF CADHERIN-11 EXPRESSION WITH THE TRANSFORMING GROWTH FACTOR-β1-MEDIATED AGGREGATION AND FUSION OF HUMAN EXTRAVILLOUS CYTOTROPHOBLASTS *IN VITRO*

## A.1.1: Preface

In these studies, we examined the ability of TGF-β1 to regulate cad-11 mRNA levels and protein expression in primary cultures of extravillous cytotrophoblasts propagated from human first trimester placental explants using Northern and Western blot analysis, respectively. TGF-β1 was capable of increasing cad-11 expression in these isolated human extravillous cytotrophoblasts. The effects of TGF-β1 on cad-11 mRNA levels and protein expression were dependent on the concentration of growth factor added to the culture medium in these trophoblastic cell cultures. Furthermore, the increase in cad-11 expression correlated with the terminal differentiation and fusion of these extravillous cytotrophoblasts *in vitro*. We believe that this is the first demonstration of a growth factor, namely TGF-β1, that is capable of regulating cad-11 mRNA levels and protein expression in a mammalian cell. The results of these studies are published in a manuscript entitled "Regulated expression of cadherin-11 in human extravillous cytotrophoblasts undergoing aggregation and fusion in response to transforming growth factor-β1" in the *Journal of Reproduction and Fertility* (S Getsios, GTC Chen, DTK Huang, and CD MacCalman (1998); 114:357-363).



GTC Chen prepared the cell lysates for the Western blot analyses in these studies. DTK Huang was an undergraduate summer student that aided in the propagation of the primary cell cultures used in these studies. The remainder of the research was conducted by S Getsios under the supervision of CD MacCalman.

## A.1.2: REGULATED EXPRESSION OF CADHERIN-11 IN HUMAN EXTRAVILLOUS CYTOTROPHOBLASTS UNDERGOING AGGREGATION AND FUSION IN RESPONSE TO TRANSFORMING GROWTH FACTOR-β1


### Abstract

TGF-β1 is believed to be a key regulator of extravillous cytotrophoblast invasion during the first trimester of pregnancy. In addition, this growth factor has been shown to regulate cellular differentiation and fusion in cultured extravillous cytotrophoblasts. To date, the cellular mechanisms by which TGF-β1 promotes these developmental processes remain poorly understood. Recent studies indicate that the expression of the novel cadherin subtype, known as cad-11, is associated with the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta *in vitro*. In this study, cad-11 mRNA and protein expression were examined in primary cultures of human extravillous cytotrophoblasts cultured in the presence of TGF-β1 using Northern and Western blot analysis, respectively. TGF-β1 was shown to increase cad-11 mRNA and protein expression in these cultured extravillous cytotrophoblasts in a dose-dependent manner. Cad-11 was further localized to the large cellular aggregates and multinucleated cells that formed in response to increasing concentrations of TGF-β1 using immunocytochemistry. Collectively, these observations suggest that the morphogenetic effects of TGF-β1 on cultured extravillous cytotrophoblasts are mediated, at least in part, by an increase in cad-11 expression. This study not only adds to the understanding of the cellular mechanisms by which TGF-β1 promotes extravillous cytotrophoblast differentiation and fusion but provides useful insight into the cell biology of the cadherins.


### Introduction

Implantation is dependent on the proliferation, differentiation, and invasion of fetal trophoblast cells into the maternal endometrium (Aplin, 1991; Strickland, 1992; Burrows *et al.*, 1996). After invasion of the uterine epithelium and underlying stroma, the trophoblasts proliferate and differentiate to form chorionic villi. The chorionic villi of the human placenta are composed of an inner cell layer of mitotically active mononucleate cytotrophoblasts, most of which undergo terminal differentiation and fusion to form the overlying syncytial trophoblast. As pregnancy proceeds, the villous cytotrophoblasts at the tips of the chorionic villi proliferate and rupture the syncytial trophoblast, forming columns that extend into the decidua. These extravillous cytotrophoblast columns are believed to anchor the placenta to the decidua. Cytotrophoblasts also dissociate from the extravillous columns and invade the maternal vasculature and decidua. These invasive cytotrophoblasts subsequently undergo differentiation and fusion to form placental bed giant cells, large multinucleated cells that lie in intimate contact with the surrounding decidual cells.

Growth factors and cytokines are believed to be key regulators of trophoblast differentiation and invasion (Tabibzadeh, 1994; Lala and Hamilton, 1996). For example, the localization of TGF-β1 to the decidual cells of the human endometrium and the syncytial trophoblast and extravillous cytotrophoblast columns of the first trimester placenta has led to the proposal that this growth factor regulates the invasive capacity of trophoblasts in both an autocrine and paracrine manner (Graham *et al.*, 1992; Lysiak *et al.*, 1995). Furthermore, TGF-β1 has been shown to reduce proliferation and promote the differentiation and fusion of highly invasive extravillous cytotrophoblasts into multinucleated cells *in vitro* (Graham and Lala, 1991; Graham *et al.*, 1992). These developmental processes have been associated with a reduction in the invasive capacity of these cells. To date, the cellular mechanism(s) by which TGF- β1 regulates trophoblast differentiation and invasion remain poorly understood.

Cytotrophoblast invasion into the decidua is likely to encompass dynamic changes in cell-cell and cell-matrix interactions (Burrows *et al.*, 1996). It has been demonstrated that the differential expression of proteases (Librach *et al.*, 1991), ECM proteins (Feinberg *et al.*, 1991), and members of the family of cell-substrate adhesion molecules, known as the integrins (Damsky

*et al.*, 1992), modulate trophoblast differentiation along the invasive pathway *in vitro* and *in vivo*. In contrast, the role(s) of the gene superfamily of $Ca^{2+}$-dependent CAMs, known as the cadherins, in trophoblast differentiation and invasion remain poorly characterized.

The cadherins are integral membrane glycoproteins that mediate cell-cell interactions in a homophilic manner (Takeichi 1991,1995; Blaschuk *et al.*, 1994). The cadherins are key morphoregulators. For example, the expression of specific cadherin subtypes is tightly regulated during development and it is believed that the differential expression of these CAMs governs the developmental fate of cells (Takeichi, 1991,1995). In adults, cadherins are believed to maintain the integrity of tissues. The loss and/or aberrant expression of cadherins mediates the neoplastic transformation of cells and facilitates tumour cell invasion (Takeichi, 1995). In view of these observations, it would seem likely that cadherins play a key role in trophoblast invasion by mediating trophoblast differentiation and/or trophoblast-endometrial cell interactions.

Cad-11 is a novel cadherin subtype that is spatiotemporally expressed in the human endometrium and placenta (MacCalman *et al.*, 1996a, 1997a). In particular, cad-11 has been localized to the syncytial trophoblast and extravillous cytotrophoblast columns of the first trimester placenta and the decidual cells of the human endometrium. As cad-11 expression is restricted to the two types of trophoblasts that form intimate contacts with these uterine cells, it is hypothesized that cad-11 mediates trophoblast-decidual cell interactions in a homophilic manner. These cellular interactions may assist in anchoring trophoblasts to decidual cells, thereby arresting their invasive migration. In addition, cad-11 expression is tightly regulated during trophoblast differentiation *in vitro* (MacCalman *et al.*, 1996a). Low concentrations of cad-11 were detected in mononucleate cytotrophoblasts isolated from the human term placenta. As the cytotrophoblasts underwent differentiation and fusion to form syncytium, there was a marked increase in cad-11 expression. Finally, cad-11 was not detected in invasive extravillous cytotrophoblasts propagated from first trimester placental explants (MacCalman *et al.*, 1998). Collectively, these observations suggest that cad-11 plays a key role in the terminal differentiation and fusion of cytotrophoblasts, and that failure to express this CAM results in the development of an invasive phenotype.

In the present study, cad-11 expression was examined in extravillous cytotrophoblasts undergoing cellular differentiation and fusion in response to TGF-β1. TGF-β1 increased cad-11 mRNA and protein expression in these primary cell cultures in a dose-dependent manner. The increase in cad-11 expression correlated with the formation of large cellular aggregates and the appearance of multinucleated cells in these primary cell cultures. Subsequently, cad-11 was localized to both of these cellular structures, using immunocytochemistry.

## Materials and Methods

### *Tissues*

First trimester placental tissues (8 to 13 weeks of gestation) were obtained from women undergoing elective termination of pregnancies. The use of these tissues was approved by the Committee for Ethical Review of Research involving Human Subjects, University of British Columbia. All subjects provided informed consent for these studies.

### *Cell Preparation and Culture*

Extravillous cytotrophoblasts were propagated from first trimester placental explants as described by Graham *et al.* (1992). Briefly, chorionic villi were washed thoroughly in DMEM (Gibco BRL, Burlington, ON) containing 25 mM glucose, 25 mM HEPES and 50 μg/ml gentamicin. The villi were minced finely and plated in 25 $cm^2$ tissue culture flasks containing DMEM supplemented with antibiotics and 10% heat-inactivated FCS. The fragments of chorionic villi were allowed to adhere for 2-3 days, after which the nonadherent material was removed. The villous explants were cultured for a further 10-14 days, with the culture medium being replaced every 3 days. The propagated extravillous cytotrophoblasts were separated from the villous explants by a brief (2-3 min) trypsin digestion (0.125% trypsin-EDTA in $Ca^{2+}$, $Mg^{2+}$-free PBS) at 37 °C and plated in 60 mm culture dishes containing DMEM supplemented with antibiotics and 10% FCS. The purity of the extravillous cytotrophoblast cultures was determined

by cytokeratin immunostaining. Only cultures which were 100% positive for cytokeratin immunostaining were used in these studies.

Extravillous cytotrophoblasts (passage 2) that had been cultured in DMEM containing 10% FCS, were washed with PBS and then cultured in serum-free DMEM for a further 24 h. This culture medium was removed and, after the cells had been washed twice with PBS, replaced with serum-free DMEM containing pig TGF- β1 (1 pg-10 ng/ml; Sigma Chemical Co., St Louis, MO) or vehicle (40 μM HCl/ 1 μg/ml BSA). The concentrations of TGF- β1 used in these experiments were selected on the basis of previous studies (Graham *et al.*, 1992; Graham, 1997). The cells were harvested for Northern or Western blot analysis or fixed for morphological assessment and immunocytochemistry after 24 h of culture in the presence or absence of TGF- β1.

*Northern Blot Analysis*

Total RNA was prepared from the cytotrophoblast cell cultures by the phenol-chloroform method of Chomczynski and Sacchi (1987). The RNA species were resolved by electrophoresis in 1% agarose gels containing 3.7% formaldehyde. Approximately 20 μg of total RNA were loaded per lane. The fractionated RNA species were then transferred onto charged nylon membranes (Amersham Canada Ltd., Oakville, ON).

The Northern blots were incubated in a 3% solution of BSA dissolved in 5 X SSPE (20 X SSPE consists of 0.2 M sodium phosphate monobasic, pH 7.4 containing 25 mM EDTA and 3 M NaCl) at 37 $^{o}$C for 30 min. The Northern blots were then transferred to a prehybridization solution consisting of 50% deionized formamide, 5 X Denhardt's solution (5 Prime, 3 Prime Inc., Boulder CO), 5 X SSPE, 5 X dextran sulfate (5 Prime, 3 Prime Inc., Boulder CO), 1% SDS, 50 mM sodium phosphate dibasic, and 5 mM sodium phosphate monobasic. The blots were

incubated in this solution at 37 °C for 2 h. Heat denatured salmon sperm DNA (final concentration 0.2 mg/ml; 5 Prime, 3 Prime Inc.) and a radiolabeled human cad-11 cDNA probe were then added to the pre-hybridization solution. The cDNA probe used in these studies is described in detail by MacCalman *et al.* (1996a). The probe was radiolabeled according to the methods of Feinberg and Vogelstein (1983) and heat-denatured, before being added to the prehybridization solution. The blots were incubated in the presence of the radiolabeled cDNA probe at 37 °C for 16 h.

The Northern blots were washed twice with 2 X SSPE at RT (10 min per wash), twice with 2 X SSPE containing 1% SDS at 55 °C (30 min per wash) and twice with 0.2 X SSPE at RT (30 min per wash). The blots were subjected to autoradiography to detect the hybridization of the radiolabeled cDNA probe to the cad-11 mRNA species. The Northern blots were also probed with a radiolabeled synthetic oligonucleotide specific for 18S rRNA according to the protocols described by MacCalman *et al.* (1992) to standardize the amounts of total RNA in each lane. The blots were again subjected to autoradiography to detect the hybridization of the radiolabeled probe to the 18S rRNA. The autoradiograms were then scanned using an LKB laser densitometer. The absorbance values obtained for the cad-11 mRNA transcript were normalized relative to the corresponding 18S rRNA absorbance value.

*Western Blot Analysis*

For Western blot analysis, the trophoblasts were incubated in 150 µl of cell lysis buffer (Tris-HCl, pH 7.5 containing 0.5% NP-40, 0.5 mM $CaCl_2$ and 1.0 mM PMSF) at 4 °C for 30 min on a rocking platform. The cell lysates were centrifuged at 10,000 x g for 20 min and the supernatant used in the Western blot analyses. Protein concentration in the cell lysates was determined using the BCA kit (Pierce Chemicals, Rockford, IL). Aliquots (30 µg) were then taken from the total cell lysates and subjected to SDS-PAGE under reducing conditions, as

described by Laemmli (1970). The stacking gels contained 5% acrylamide and the separating gels were composed of 7.5% acrylamide. The proteins were electrophoretically transferred from the gels onto nitrocellulose paper according to the procedures of Towbin *et al.* (1979). The nitrocellulose blots were probed with a mouse monoclonal antibody directed against human cad-11 (C11-113H; ICOS Corporation, Bothell, WA) as previously described by MacCalman *et al.* (1996a) and Getsios *et al.* (1998a). The Amersham ECL system was used to detect antibody bound to antigen. The autoradiograms were then scanned using an LKB laser densitometer.

*Immunocytochemistry*

The cells were washed twice in PBS and fixed in 4% paraformaldehyde at 4°C for 20 min. Immunocytochemistry was performed using a mouse monoclonal antibody directed against human cytokeratin filaments 8 and 18 (Becton Dickson, San Jose, CA) or a monoclonal antibody directed against human cad-11 (C11-113E; ICOS Corporation, Bothell, WA) as described by MacCalman *et al.* (1996a, 1997a) and Getsios *et al.* (1998a). A nonspecific isotype-matched mouse $IgG_{2a}$ monoclonal antibody (Dako Corp., Carpenteria, CA) was used as a negative control. Sequential incubations were performed according to the methods of Cartun and Pedersen (1989) and included 10% normal horse serum for 30 min, primary antibody at 37°C for 1 h, secondary biotinylated antibody at 37°C for 45 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three 5 minute washes in PBS. The cells were then exposed to chromagen reaction solution (0.035% diaminobenzidine and 0.03% $H_2O_2$) for 10 min, washed in tap water for 5 min, counterstained in haematoxylin, dehydrated, cleared, and mounted.

*Statistical Analysis*

The results are presented as the mean relative absorbance (± SEM) for three independent experiments. Statistical differences between treatments were assessed by ANOVA. Differences

were considered to be significant for $P \leq 0.05$. Significant differences between the means were determined using the least significant difference test.

## Results

A single cad-11 mRNA transcript of 4.4 kb was detected in all of the total RNA extracts prepared from the cultured extravillous cytotrophoblasts (Fig. A.1.2.1). TGF-β1 increased cad-11 mRNA in the cultured extravillous cytotrophoblasts in a dose-dependent manner. However, a significant increase in cad-11 mRNA was only observed in extracts prepared from trophoblasts cultured in the presence of 1 or 10 ng/ml of TGF-β1. Maximum cad-11 mRNA concentrations were observed in extravillous cytotrophoblasts cultured in the presence of 10 ng/ml TGF-β1.

Western blot analysis, using extracts prepared from extravillous cytotrophoblasts cultured in the presence or absence of TGF-β 1 and a mouse monoclonal antibody directed against human cad-11, revealed a single cad-11 protein species ($M_r$ 125 kDa) in all of the cellular extracts (Fig. A.1.2.2). In agreement with the Northern blot analysis, TGF-β1 caused an increase in cad-11 expression in a dose-dependent manner with a significant increase in cad-11 protein expression only being observed in cytotrophoblasts cultured in the presence of 1 or 10 ng/ml of TGF-β1. Maximum cad-11 expression was observed in extracts prepared from cells cultured in the presence of 10 ng/ml TGF-β1.

Finally, extravillous cytotrophoblasts cultured in the presence or absence of TGF- β1 for 24 h were immunostained for cad-11. There was marked increase in the number of cells expressing cad-11 in cultures treated with 1 or 10 ng/ml of TGF-β1 but not in cultures treated with lower concentrations of TGF-β1 (Fig. A.1.2.3). Cad-11 was further localized to the large cellular aggregates and multinucleated cells which were observed primarily in cultures treated with 1 or 10 ng/ml of TGF-β1. These cellular structures were predominant in cultures treated with 10 ng/ml TGF-β1.

## Discussion

The mechanisms by which TGF-β1 regulates trophoblast differentiation and invasion remain poorly understood. Previous studies have demonstrated that TGF-β1 is capable of increasing the production of protease inhibitors in cultured extravillous cytotrophoblasts in a dose-dependent manner (Graham and Lala, 1991; Graham, 1997). These observations have led to the proposal that TGF-β1 may regulate trophoblast invasion and migration by modulating the proteolytic activity of these cells. In addition, the ability of TGF-β1 to regulate the expression of oncofetal Fn (Feinberg *et al.*, 1994) and specific integrin subunits (Irving and Lala, 1995) in trophoblasts suggests that this growth factor is capable of modulating the adhesive mechanisms likely to be involved in trophoblast invasion. In the present study, we have demonstrated that cad-11 expression is up-regulated in extravillous cytotrophoblasts undergoing aggregation and fusion in response to increasing concentrations of TGF-β1. In view of these observations, it is possible that cad-11 plays a central role in trophoblast invasion by promoting cellular interactions in a homophilic manner and mediating the differentiation and fusion of these cells.

We have previously failed to detect significant cad-11 expression in extravillous cytotrophoblasts propagated from first trimester placental explants (MacCalman *et al.*, 1998). These trophoblasts are highly invasive and form few cellular interactions with one another. In contrast, cad-11 was readily detectable in the cellular aggregates that formed in response to increasing concentrations of TGF-β1. Similarly, cad-11 was detected in the trophoblast aggregates that constitute the extravillous cytotrophoblast columns of the first trimester placenta (MacCalman *et al.*, 1996a). Collectively, these observations suggest that cad-11 mediates trophoblast-trophoblast interactions in a homophilic manner. These cad-11-mediated cellular interactions may be a prerequisite for the fusion of extravillous cytotrophoblasts *in vitro* and may play a key role in maintaining the integrity of extravillous cytotrophoblast columns *in vivo*. The subsequent loss or failure to express this CAM may allow trophoblasts to dissociate from the extravillous column and invade into the underlying maternal tissues.

Cad-11 was also detected in the multinucleated cells that formed in response to TGF-β1, suggesting that this CAM not only mediates cellular interactions but also plays a key role in the

terminal differentiation and fusion of cultured extravillous cytotrophoblasts. Cad-11 expression has been shown to be regulated tightly during the terminal differentiation and fusion of several other cell types. For example, cad-11 expression has been shown to increase during the terminal differentiation of human endometrial stromal cells (MacCalman *et al.*, 1996a) and osteoblasts (Okazaki *et al.*, 1994). Furthermore, there is a marked increase in cad-11 expression during the differentiation and fusion of trophoblasts isolated from the human term placenta and in BeWo choriocarcinoma cells undergoing differentiation and fusion in response to cAMP (MacCalman *et al.*, 1996a). To date, the role of cad-11 in cellular differentiation and fusion has not been defined.

Recent studies indicate that trophoblasts and carcinoma cells use the same adhesive mechanisms during cellular invasion (Tang and Honn, 1994; Lala and Hamilton, 1996; Vicovac and Aplin, 1996). The regulated expression of members of the Ig gene superfamily, integrin subunits, and cadherins in carcinoma cells and extravillous cytotrophoblasts are believed to promote cellular invasion and facilitate the interactions of these invasive cells with the ECM and cells of the stroma and vasculature. Consistent with these observations, Shibata *et al.* (1996) have detected cad-11 mRNA transcripts in signet-ring cell carcinoma cells and the surrounding stromal cells, suggesting that this CAM may mediate carcinoma-stromal cell interactions. Similarly, the spatiotemporal expression of cad-11 in the human placenta and endometrium has led us to propose that this CAM mediates trophoblast-decidual cells interactions in a homophilic manner. Finally, as the formation of syncytium in extravillous cytotrophoblast cultures treated with TGF-β1 is believed to reflect the formation of placental bed giant cells (Graham *et al.*, 1992), large multinucleated cells that lie embedded in the decidua, it is possible that cad-11 may also mediate cellular interactions between these trophoblast cells and the surrounding decidual cells. Collectively, these observations strengthen our hypothesis that cad-11-mediated cellular interactions play a central role in regulating trophoblast invasion.

In summary, cad-11 expression was shown to be associated with the aggregation and fusion of extravillous cytotrophoblasts in response to TGF-β1. These observations add to our understanding of the cellular mechanisms by which TGF-β1 inhibits trophoblast differentiation

along the invasive pathway and provide useful insight into the cell biology of this novel cadherin subtype.

Fig. A.1.2.1: (a,b) Autoradiograms of a Northern blot containing total RNA extracted from extravillous cytotrophoblasts cultured in the presence of vehicle, 0.001, 0.01, 0.1, 1, or 10 ng/ml of TGF-β1 for 24 h (lanes 1-6, respectively). The blot was probed for cad-11 (a) or 18S rRNA (b).

(c) The autoradiograms were scanned using a laser densitometer. The values obtained for the cad-11 mRNA transcript were then normalized to the absorbance values obtained for the corresponding 18S rRNA. The results derived from this analysis, as well as from two other studies (autoradiograms not shown) were standardized to the vehicle control (mean $\pm$ SEM; n=3, $*P \leq 0.05$).

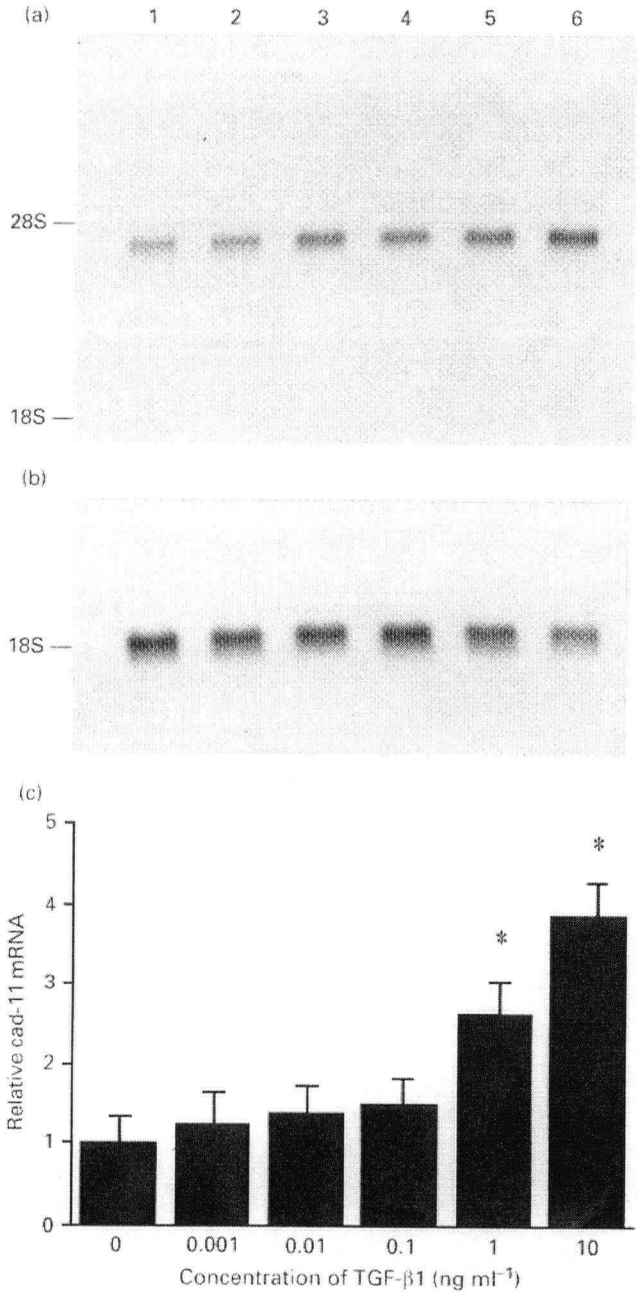

(a)
1
2
3
4
5
6
28S
18S
(b)
18S
(c)
Relative cad-11 mRNA
0
1
2
3
4
5
*
*
0
0.001
0.01
0.1
1
10
Concentration of TGF-β1 (ng ml⁻¹)

Fig. A.1.2.2: (a) Western blot analysis of cad-11 expression levels in cultured extravillous cytotrophoblasts. Protein (30 μg) extracted from cells cultured in the presence of vehicle, 0.01, 0.1, 1, or 10 ng/ml of TGF-β1 for 24h (lanes 1-5, respectively) were loaded in each lane of an SDS-polyacrylamide gel. Western blot analysis was performed using a mouse monoclonal antibody directed against human cad-11. The Amersham enhanced chemiluminescence (ECL) system was used to detect antibody bound to antigen.

(b) The autoradiograms were then scanned using an LKB laser densitometer. The results derived from this analysis, as well as from two other studies (autoradiograms not shown) were standardized to the vehicle control (mean $\pm$ SEM; n=3, $^{*}P \leq 0.05$).

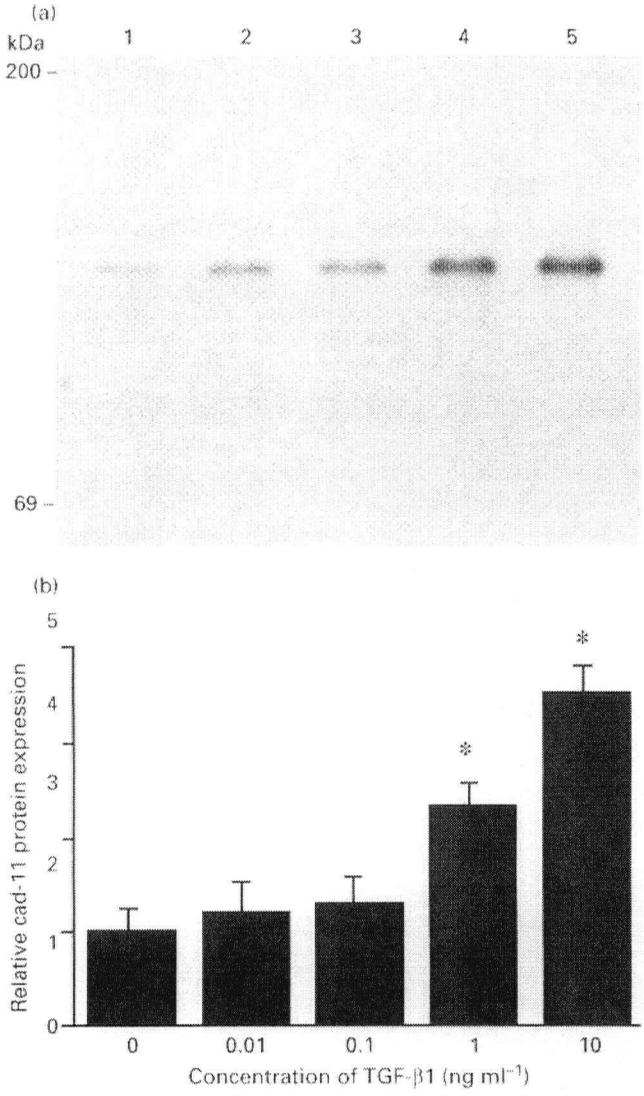
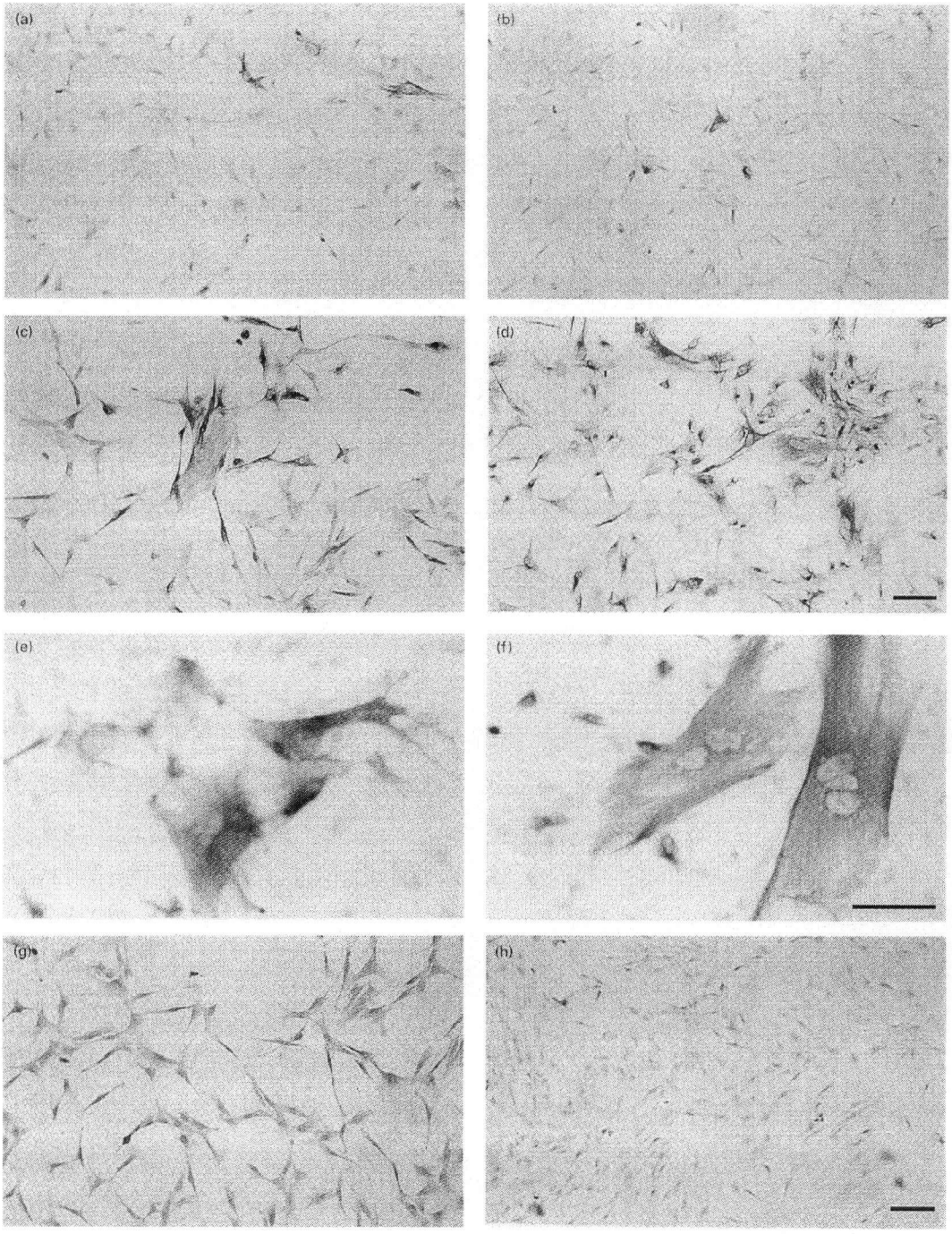
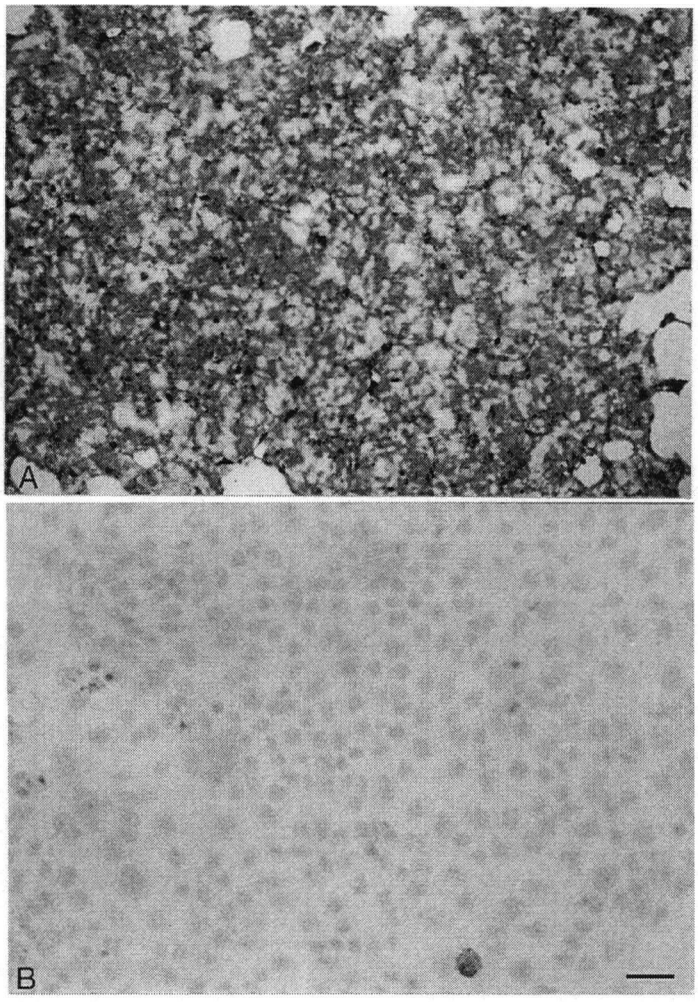

(a)
kDa
1
2
3
4
5
200
69
(b)
Relative cad-11 protein expression
0
1
2
3
4
5
*
*
0
0.01
0.1
1
10
Concentration of TGF-β1 (ng ml⁻¹)

Fig. A.1.2.3: Immunodetection of cad-11 in extravillous cytotrophoblasts cultured in the presence of vehicle alone, 0.1, 1, or 10ng/ml TGF-β1 for 24 h (panels a-d, respectively). Cad-11 immunostaining was localized to the large cellular aggregates (panel e) and multinucleated cells (panel f) which formed in response to increasing concentrations of TGF-β1. (g) The purity of the extravillous cytotrophoblasts used in these studies was determined by immunostaining for cytokeratin (panel g). Negative control in which the primary antibody was replaced with a nonspecific mouse monoclonal antibody (panel h). Scale bar represents 50 μm.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

## APPENDIX II: X-GAL CYTOCHEMISTRY IN JEG-3 CELLS TRANSFECTED WITH p*LACZ* OR pCAD-11.

The transfection efficiency of p*lacZ* in JEG-3 cell cultures was determined using X-gal cytochemistry. Photomicrographs of X-gal cytochemistry in JEG-3 cells transfected with p*lacZ* or pCAD-11 and cultured for and additional 24 h (panels A and B, respectively). The cells were fixed in 2% paraformaldehyde/0.2% glutaraldehyde for 15 min at RT. To detect β-galactosidase gene expression in these cultures, the fixed cells were stained with the substrate for this enzyme, X-gal, as previously described by MacCalman *et al.* (1996b). The scale bar represents 40 μm.


A
B