GAP JUNCTIONS IN EARLY HUMAN PLACENTAL DEVELOPMENT

by

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Gap junctional intercellular communication (GJIC) is known to mediate cell proliferation, differentiation, and invasion. We hypothesize that GJIC plays a role in early human placental development. By RT-PCR, we identified transcripts for Cx32, Cx37, Cx40, Cx43, and Cx45 in the first trimester human placenta. Of these, Cx40 mRNA and protein were prominent in anchoring extra-villous trophoblast (EVT) columns. Inhibition of GJIC in first trimester placental villous explant cultures by treatment with carbenoxolone and heptanol induced a switch from a proliferative EVT phenotype to an early invasive phenotype, as evident from: a) a loss of the proliferation marker Ki67, and b) a decrease in matrix-metalloproteinase-2 activity (which is normally present in proliferative EVT columns). Furthermore, the morphology of EVT outgrowths changed dramatically upon GJIC-blockade from compact, organized outgrowths to a scattered group of individual EVT cells, reminiscent of an early invasive phenotype. Since Cx40 is predominant in anchoring columns *in vivo*, we treated placental villous explants with antisense oligonucleotides to Cx40. This resulted in identical changes in EVT outgrowth morphology as the non-specific GJIC inhibitors. Together, our data suggest that the presence of functional GJIC, particularly through Cx40 channels, is necessary for the establishment of proliferative EVT cell columns in the early human placenta, and that a loss of GJIC may contribute to the switch to the invasive trophoblast phenotype.

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CONTRIBUTIONS

The following persons contributed to results presented in this thesis:

Chapter 2

- Whole-mount *in situ* hybridization experiments shown in Figure 2.7 were performed in collaboration with Yong Lu (CIHR Histology Core Research Associate, Samuel Lunenfeld Research Institute).

Chapter 3

- Micro-injections of placental villous explant EVT cells were performed by Xu Feng (Dr. L. Langille's lab, Toronto General Hospital), Figure 3.2.

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LIST OF ABBREVIATIONS

AV	atrial-ventricular
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
CBX	carbenoxolone
cDNA	complementary deoxynucleic acid
CTB	cytotrophoblast
Cx	connexin
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
d-UTP	deoxy-uracil tri-phosphate
EGF	epidermal growth factor
EVT	extra-villous trophoblast
GJIC	gap junctional intercellular communication
GLUT1	glucose transporter-1
hCG	human chorionic gonadotropin
HGF	hepatocyte growth factor
HIF-1	hypoxia-inducible factor-1
HLA-G	human leukocyte antigen-G
hPL	human placental lactogen
11β-HSD-2	11β-hydroxysteroid dehydrogenase-2
ICM	inner cell mass
IGF	insulin-like growth factor
IL	interleukin
IUGR	intrauterine growth restriction
MMP	matrix-metalloproteinase
NO	nitric oxide
NOS	nitric oxide syntase
PBS	phosphate buffered saline
pc	post-coitus
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKA	protein kinase A
PKC	protein kinase C
PlGF	placental growth factor

pVHL	von Hippel-Lindau protein
RNA	ribonucleic acid
ROD	raw optical density
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
STB	syncytiotrophoblast
TAE	Tris acetate-EDTA
TBS-T	Tris buffered saline-Tween 20
TGF-β	transforming growth factor-β
TIMP	tissue inhibitors of metalloproteinase
tRNA	transfer ribonucleic acid
U	units
VEGF	vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

Literature Review

The Placenta

1.1 Introduction

The placenta is a specialized organ that develops and functions only during pregnancy. Traditionally thought to serve solely as an interface for the exchange of nutrients, oxygen, and waste products between the mother and fetus, the placenta is now known to synthesize a myriad of hormones, enzymes, and bioactive peptides (1), thus also serving complex endocrine and paracrine roles throughout pregnancy. The proper functioning of the placenta is critical for both normal fetal development, and for maternal adaptations that are required to maintain pregnancy. Several clinical conditions are associated with perturbations of placental function, such as miscarriage in the first trimester and the third-trimester complications of pre-eclampsia, intrauterine growth restriction (IUGR) and abruption (premature placental separation) (2). Moreover, the relative size of one's placenta at birth has been implicated in the adult onset of cardiovascular disease and diabetes; the larger the ratio of placental weight to newborn weight when born, the greater the likelihood of developing these diseases later in life (3). The development of the human placenta is a very complex process, involving transcription factors, growth factors/cytokines, matrix-metalloproteinases, integrins and cadherins, and changes in oxygen tension, all of which will be discussed in further detail in this chapter. Recent evidence also suggests that communication between cells via gap junctions may play a role in placental development, but additional research is needed to fully understand the relevance of gap junctional communication during human placentation. Therefore, the **overall aims of this thesis project** were to assess the expression patterns of gap junctions in the early human placenta, and to investigate the possible role of gap junctional communication in early placental differentiation.

The placenta is a transient tissue that must form very early in development in order to facilitate the growth of the embryo. The placental lineage is the first to differentiate following fertilization in all viviparous animals, when the trophectoderm differentiates to surround the inner cell mass (ICM) in the early blastocyst (2). Species variations in placentation arise soon after this initial differentiation process, and mature placentas from different species have drastically different structures and functions. For example, in primates and rodents, maternal

uterine vessels are breached by trophoblast cells; as a result, maternal blood is in direct contact with trophoblast cells. In the pig, on the other hand, there is no invasion, and trophoblasts are apposed to the maternal uterine epithelium throughout the course of pregnancy (4). Interestingly, no relationship seems to exist between placental classification and taxonomy (5). Variances of placental structure can make placental research difficult, since cross-species comparisons must be made with caution. Therefore, this project focused on human placental development.

1.2 Placental Development

There has been extensive study of the human placenta, especially since term tissue samples are readily available following childbirth. The anatomical changes that occur during early human placental development have also been well characterized (6), and clues into molecular mechanisms regulating human placentation have more recently been extrapolated from studies of the mouse placenta. The following sub-section will describe human placental development in terms of anatomy and structure, and a comparison with mouse placental development will follow, since the mouse model is becoming a powerful tool to dissect molecular developmental pathways in the placenta.

1.2.1 Human Placental Development

At the time of implantation, which occurs at day 6 to 7 post-coitus (pc) in the human, the blastocyst is composed of an ICM surrounded by a layer of trophectoderm, which eventually gives rise to extra-embryonic tissues, including the placenta (7). The ICM comprises a small group of larger cells that become the embryo proper as well as the umbilical cord and amnion. The blastocyst orientates itself upon implantation so that the embryonic pole (the part that bears the ICM at its inner surface) is attached to the maternal endometrium first. Once the blastocyst has invaded the endometrial epithelium, the trophoblastic cells of the implanting embryonic pole undergo increased proliferation, which results in a double-layered trophoblast. The outermost layer fuses to form the syncytiotrophoblast (STB), whereas the underlying cytotrophoblast cells (CTBs) remain unfused and proliferative, serving as stem cells for the STB layer. The STB layer becomes a thick mass, and by day 8 pc, small vacuoles appear in this mass, expanding to form a system of *lacunae* (refer to Figure 1.1, A). The blastocyst becomes so deeply implanted in the

maternal uterine epithelium that by day 12 pc the epithelium closes over the implantation site. The trophectoderm that is not adjacent to the ICM does not proliferate and ultimately differentiates into the smooth chorion that surrounds the rest of the embryo.

CTBs begin to invade the syncytial trabeculae at around day 12 pc, and within a few days CTBs line the entire length of the trabeculae, giving rise to primitive primary villous structures (Figure 1.1, A). The lacunar spaces become the intervillous space, which will eventually be filled with maternal blood. Soon after the CTBs line the syncytial trabeculae, fetally-derived mesenchymal cells invade the villi centrally, transforming them into secondary villi (Figure 1.1, B). If these villi remain in contact with the maternal decidua, they are termed anchoring villi; if they do not they are termed floating villi, since they float in the intervillous space. By day 20 pc, the first fetal capillaries in the mesenchyme of placental villi can be observed. They are derived from hemangioblastic progenitor cells, which differentiate locally from the mesenchyme. The presence of capillaries matures the villi into tertiary villi (composed of three main cell-types: trophoblast, mesenchyme, and endothelial cells) (Figure 1.1, C). A complete fetoplacental circulation is established at around the beginning of the 5th week pc, as soon as enough capillary segments fuse to form a capillary bed within the villi.

The branching of early villous trees occurs when local CTBs proliferate at the surfaces of larger villi, fuse into syncytium, and are invaded by villous mesenchyme, thus being transformed into villous sprouts. These grow in length and width and develop capillaries, resulting in a new branch in the villous tree. In the first trimester, these villi are composed of both an outer STB and an underlying proliferative CTB layer. Throughout the second and third trimesters, however, the CTB layer becomes discontinuous because these stem cells stop proliferating and by term CTBs are found at only 20% of villous surfaces. Once the placenta is formed, maternal blood components are transferred through multiple layers from the intervillous space to fetal villous capillaries: the STB layer, the underlying villous CTB (where present), the villous stroma and finally the villous capillary endothelium. Because of the direct interaction of maternal blood with the STB layer of the placenta, the human placenta is called haemochorial.

Throughout pregnancy, the placenta serves significant endocrine functions, producing steroids, hormones, and bioactive peptides that modulate both maternal and fetal physiological function. The placenta produces estrogens, progesterone, and mineralocorticoids, the latter of

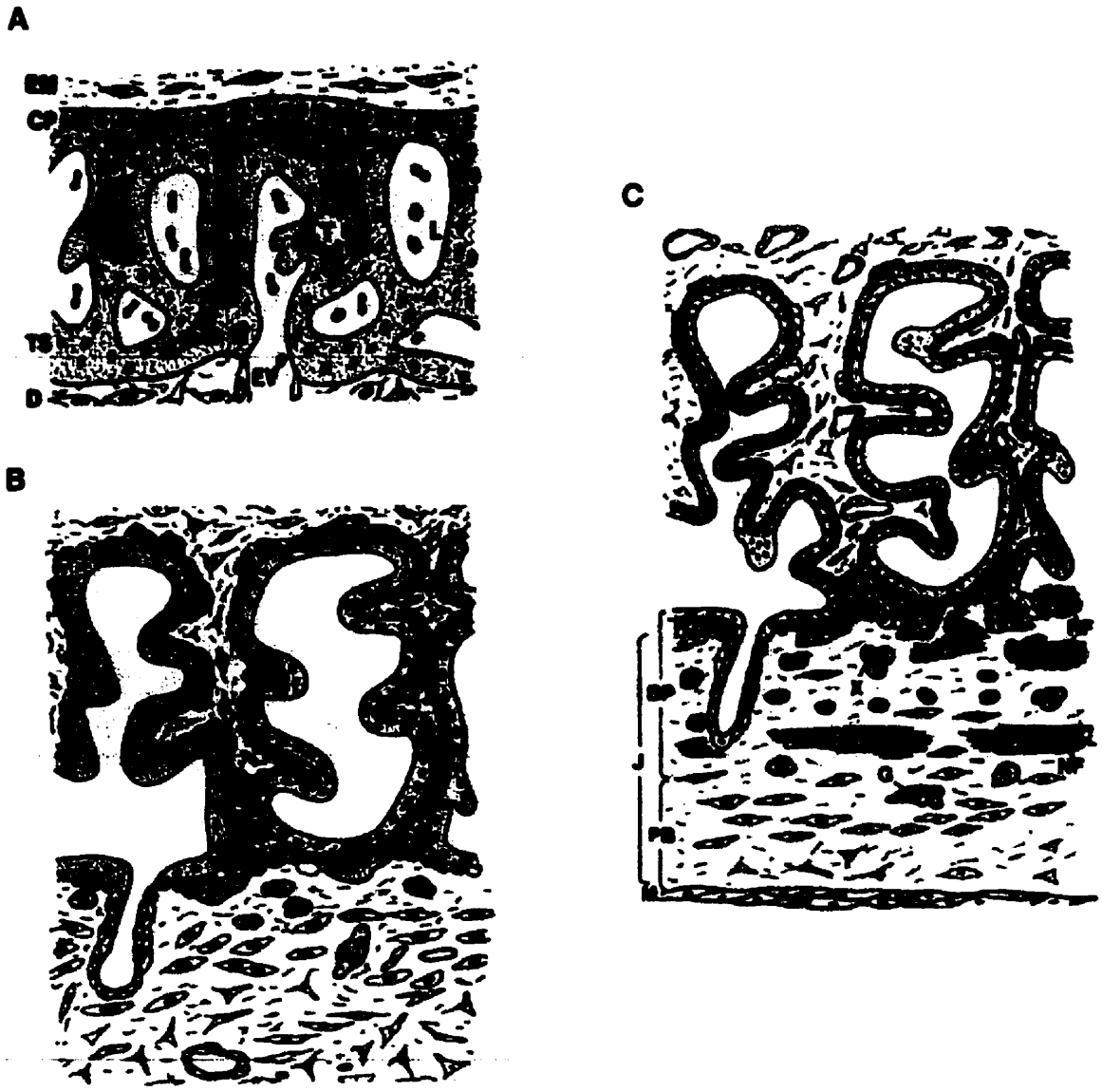

Figure 1.1. Early human placental development. Diagrammatic representation of early human placental development showing transformation of primary villous sprouts (A) into secondary villi (B) and tertiary villi (C). (EM=extraembryonic mesoderm, CP=chorionic plate, T=trabeculae and primary villi, TS=syncytiotrophoblast, D=decidua, L=maternal blood lacunae, EV=endometrial vessel, J=junctional zone, BP=basal plate, PB=placental bed, M=myometrium, X=EVT cell, G=trophoblast giant cell) (Adapted from Kingdom and Kaufmann[2]). See text on page 4 for further details.

which then act maternally to increase plasma levels of renin, angiotensinogen, and angiotensin II (8). During human pregnancy there is a vast daily production of human placental lactogen (hPL) and human chorionic gonadotropin (hCG). Other products of the human placenta include chorionic adrenocorticotropin (cACTH), human chorionic thyrotropin (hCT), luteinizing hormone releasing hormone (LHRH), corticotropin-releasing hormone (CRH), and somatostatin (8). These hormones are primarily synthesized in the STB layer of the human placenta. Although all are essential for normal human pregnancy, none of these hormones are thought to directly influence the process of trophoblast differentiation in early placental development, which is the focus of this project. Therefore, they will not be discussed further.

In addition to differentiating into syncytium to form the villous structure of the human placenta, CTBs can also differentiate down the extra-villous cytotrophoblast (EVT) pathway. Little is currently known about the mechanisms by which a villous CTB stem cell is diverted from the syncytial to the EVT differentiation pathway. EVT cells can be found in various locations in the placenta, the most important and well studied being those of anchoring cell columns, since they differentiate into invasive CTB cells, which invade deep into the maternal decidua to mediate the transformation of maternal spiral arteries (9). This process, known as interstitial trophoblast invasion, is required for trophoblasts to replace endothelial cells surrounding maternal arteries. This in turn results in the increased diameter of these resistance vessels, allowing for an increase in blood flow to the intervillous space in the placenta and hence better maternofetal exchange of blood components (10). By the late second trimester of pregnancy, maternal spiral arteries are lined exclusively by CTBs, and endothelial cells are no longer visible in either the endometrial or the superficial portions of their myometrial segments (11). Further details of EVT differentiation along the invasive pathway will be discussed in section 1.4, since this differentiation pathway was examined in this project.

1.2.2 Rodent Placental Development

The initial phases of rodent placental development are identical to that of the human, in that the trophectoderm differentiates around the ICM and implantation of the blastocyst into the decidua occurs (12). However, the mature rodent placenta is composed of different cell-types arranged in a slightly different structure compared to that of the human. The adult mouse placenta contains three trophoblast cell types: the innermost labyrinthine layer, the intermediate

spongiotrophoblast and an outermost layer of trophoblast giant cells (13). Functionally, the labyrinthine layer is analogous to the floating villi of the human placenta, but instead of the villous tree structure it contains highly folded and branched layers of two syncytium (STB I and II). Trophoblast giant cells are similar to invasive human EVT cells, in that they are both polyploid and both interact with maternal blood vessels, although giant cells are not obviously invasive *in vivo* (12). The molecular mechanisms of giant cell differentiation in the mouse have been investigated extensively by the generation of mice with null mutations in various genes, which has greatly advanced the understanding of human EVT differentiation towards the invasive phenotype.

1.3 Mouse Trophoblast Giant Cell Differentiation

In the mouse placenta, trophoblast giant cells are the first cells of the trophoblast lineage to differentiate (14). They are termed giant cells because they undergo a morphological transformation following rounds of DNA synthesis in the absence of intervening mitoses, a process called endoreduplication (15). 'Primary' giant cells are formed from the trophectoderm soon after mouse blastocyst implantation. Additional ('secondary') giant cells are derived from precursors that reside in the spongiotrophoblast layer (13). Giant cell differentiation from these precursors occurs under the control of a group of basic helix-loop-helix (bHLH) transcription factors. These transcription factors are known to regulate the differentiation of a variety of cell lineages, such as muscle and neurons (16, 17) by balancing the mitotic capacity of progenitor stem cell populations with the differentiative capacity of their progeny. The basic domain of these transcription factors can bind DNA in the promoter regions of genes with an appropriate concensus sequence (E-box sequence, CANNTG) (18). They form dimers with other bHLH proteins, such as E-factors, which are widely and constitutively expressed, via the HLH-dimerization domain. Furthermore, E-factor function can be inhibted by Id proteins (inhibitors of differentiation), HLH proteins that lack the basic DNA-binding domain (19).

Three bHLH transcription factors are expressed dynamically during trophoblast differentiation in the mouse: *Mash2* (14, 20), *Hand1* (21), and *Stra13* (22). Trophoblast stem cells express *Mash2*, and both *Id-1* and *Id-2*. As these cells differentiate into cells of the spongiotrophoblast, *Id-1* and *Id-2* are no longer expressed, and *Hand1* expression appears (23). To allow for differentiation towards giant cells from spongiotrophoblast, *Mash2* is down-

regulated, and *Strat3* expression appears. The changes in bHLH transcription factors in giant cell differentiation are presented in Figure 1.2. *Mash2*-deficient mice demonstrate a loss of giant cell precursors in the spongiotrophoblast layer but an increase in giant cell number. Therefore, *Mash2* must normally function to either sustain spongiotrophoblast proliferation/survival or to block giant cell differentiation from spongiotrophoblast (24). In contrast, the role of *Hand1*, as elucidated from both mice with null mutations in the *Hand1* gene and over-expression studies (21), is to induce giant cell differentiation. (In *Hand1*$^{-/-}$ mice trophoblast cells arrest at a precursor stage (25)).

Kraut *et al.* have also specified a role for *I-mfa*, a non-HLH inhibitor of the bHLH transcription factors, in placental giant cell differentiation (26). They have shown that in *I-mfa*-deficient mice there is a strong reduction in the number of giant cells, indicating that it is required for giant cell differentation. A mechanism by which it could promote differentiation is by inhibiting *Mash2* transcriptional activity, which they demonstrate in an *in vitro* transfection study.

1.4 Human Extra-Villous Trophoblast Differentiation and Invasion

Differentiation of human extra-villous trophoblast cells along the invasive pathway is essential for human placental function. These cells arise in the cell columns of anchoring villi, which form bridges to the maternal decidua. In a process that has many similarities to tumour invasion (27), the cells that emanate from these columns invade deep into the decidua (interstitial invasion). They breach and enlarge maternal spiral arteries, and to a lesser extent veins (28), which increases blood flow to the intervillous space, thereby increasing nutrient, oxygen and waste exchange across the placenta. Some EVT cells in the human placenta also become so-called placental bed giant cells once they have invaded into the decidua, which have been reported to be most numerous during mid gestation (29). Al-Lamki *et al.* have recently demonstrated the trophoblast origin of these cells by immunostaining early placental bed biopsies with the trophoblast-specific antibody cytokeratin (30). What remains unclear, however, is whether their 'giant' size is due to endoreduplication, as occurs in mouse trophoblast giant cell formation, or to EVT cell fusion. The ultrastructural examination of these cells by electron microscopy, performed in the Al-Lamki study, is strongly supportive of the fusion

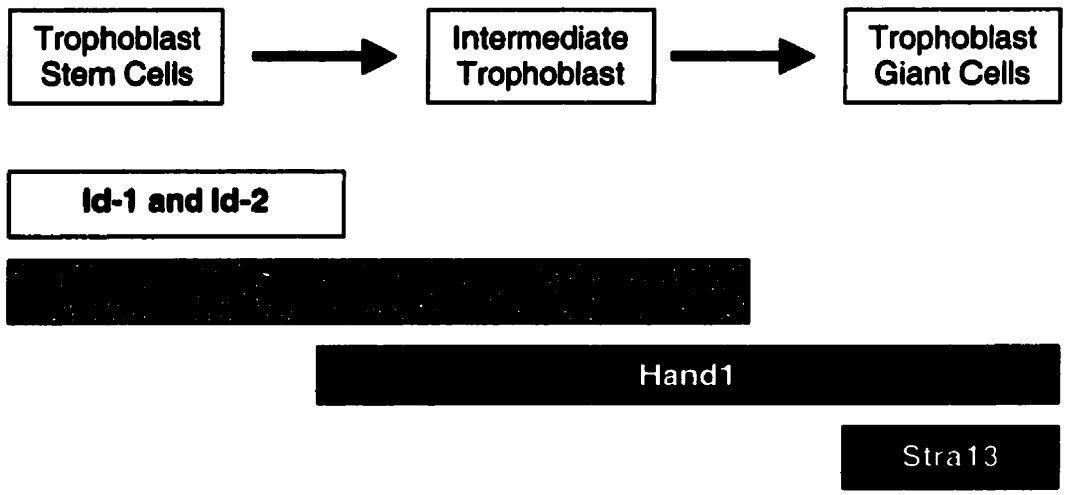

Figure 1.2. bHLH transcription factors in murine trophoblast giant cell differentiation. Trophoblast stem cells express Id-1, Id-2, and Mash2 bHLH transcription factors. In the intermediate trophoblast phenotype, Hand1 is expressed, which persists through differentiation into trophoblast giant cells. Mash2 must be down-regulated to allow for giant cell differentiation. Stra13 is expressed only in the fully differentiated giant cell.

hypothesis, although they are not conclusive, since other hallmarks of fusion (such as remnants of intracytoplasmic desmosomes and mitotic figures) were not evident.

In an anchoring villous cell column, EVT cells at the base of the column proximal to the villous tip are proliferative, as indicated by positive immunostaining with antibodies against proliferation markers such as Ki67, MIB-1, and PCNA (proliferating cell nuclear antigen) (31). Therefore, it is thought that cells close to the villous basal lamina provide the stem cell population, and all other EVT cells are more differentiated, derived from the nearest proliferative center by migration (9). CTB differentiation and invasion is a dynamic process that requires the precise spatial and temporal expression of effector proteins, which include transcription factors, growth factors and cytokines, matrix-metalloproteinases, adhesion molecules, and gap junctions. The mechanisms and markers of trophoblast differentiation along the invasive pathway will be discussed in the following sub-sections.

1.4.1 Transcription factors in Human EVT Differentiation

The elucidation of transcription factors that regulate human EVT differentiation towards the invasive phenotype has largely been based on studies of trophoblast giant cell differentiation in the mouse, as discussed in section 1.3. *Mash2*, *Stra13*, E-factor,
and *Id* genes are all expressed in the human placenta, and, as in the mouse placenta, are dynamically regulated during differentiation of EVT cells (28). A study by Alders *et al.* has demonstrated expression of *Mash2* in EVT cells *in situ*, but they did not address whether or not *Mash2* is down-regulated as differentiation progresses to the invasive stage (32). However, in studies using cultures of purified CTBs, Janatpour *et al.* have been able to demonstrate a decrease in *Mash2* as the cells become invasive (28). Furthermore, both *Id-2* and E-factor gene expressions also decrease as the cells differentiate. *Id-2* expression has also been shown to decrease *in vivo* in EVT anchoring cell columns, being down-regulated at the protein level further away from the villous tip (33). Furthermore, it has been demonstrated that the over-expression of *Id-2* in isolated trophoblast cells maintains them in an immature, undifferentiated phenotype, and inhibits the upregulation of invasive markers that usually occur with time in this culture system, such as α_1 integrin. Thus, the down-regulation of these bHLH transcription factors mirrors their changes in mouse giant cell differentiation. Similarly, the upregulation of *Stra13* during human EVT differentiation occurs during *in vitro* differentiation studies. The only

major difference is in *Hand1* expression, since it is undetectable in first trimester and term placenta (34). Janatpour *et al.* have also identified a novel human bHLH transcription factor using bioinformatics to search human EST databases (28). EST41903 is high in freshly isolated trophoblast cells, more so in first-trimester than second-trimester cells, and as these cells differentiate expression is down-regulated. They are cloning the mouse homologue of this gene to perform functional genetic analysis of this novel factor. Other transcription factors that are thought to play a major role in human EVT differentiation are the bHLH/PAS family members, HIF-1α, HIF-2α, and HIF-1β. These will be discussed in the section concerning the role of oxygen in mediating trophoblast differentiation and invasion (section 1.4.5), since these transcription factors are regulated by oxygen.

1.4.2 Growth Factors/Cytokines

The changes in transcription factor expression that occur during EVT differentiation may be due to changes in extracellular signaling molecules that activate intracellular signaling cascades to alter transcription factor expression or activation. This section will discuss growth factors and cytokines that act on EVT cells between the proliferative phenotype through to the invasive phenotype.

Epidermal Growth Factor

Epidermal growth factor (EGF) has been implicated in the regulation of trophoblast differentiation through both of the trophoblast differentiation pathways; towards syncytium and towards the invasive EVT phenotype (3). Immunolocalization of the EGF-receptor and c-erb-B2, the two closely related tyrosine kinase receptors for EGF, has revealed reciprocal expression of these receptors by non-invasive and invasive human trophoblast populations (35). King *et al.* have found that EGF-R is expressed by proliferative villous CTB, but not by non-proliferative, invasive EVT cells. In contrast, c-erbB-2 is expressed by invasive EVT cells but not by villous CTB. Expression of both is found in terminally differentiated placental bed giant cells and in villous STB (36). The spatial expression pattern suggests that EGF-R plays an important role in trophoblast proliferation whereas c-erbB2 may be important for differentiation and invasion. A role for EGF in trophoblast proliferation is supported by Li *et al.*, who treated a cell line of normal placental CTB with EGF and observed an increase in proliferation (37). In contrast,

another group has shown that the addition of EGF to first trimester CTB cultures produces a several-fold increase in invasive capacity (38). They detected EGF-R mRNA and protein on the isolated CTB cells, but failed to detect EGF ligands in CTB cells or conditioned media from the cells, which led them to speculate that maternal EGF may be acting on CTB cells *in vivo* to promote trophoblast invasion. One of the enigmas in demonstrating the role of EGF in placental development and function is the failure to consistently demonstrate the synthesis or presence of these peptides in the placenta, even though the receptors are expressed. Johki *et al.* were unable to detect EGF production by ELISA analysis in either placental or decidual cells isolated from first trimester tissues (39), whereas other groups have been able to demonstrate intense EGF staining in first trimester placenta, in both CTB and STB, with more intense staining in syncytium (40, 41).

EGF has also been shown to stimulate syncytial fusion in cultured CTB cells (42-44). In addition to morphologic changes indicative of syncytium formation, EGF treatment also increases hCG and hPL secretion, two hormones produced by STB (45). The effect of EGF in promoting trophoblast syncytialization has even been used to construct a subtractive cDNA library to identify genes associated with the *in vitro* differentiation of human CTBs towards syncytium (46). Since the focus of this thesis is EVT differentiation, details of syncytial differentiation will not be discussed further.

Vascular Endothelial Growth Factor and Placental Growth Factor

The vascular endothelial growth factor (VEGF) family includes VEGF-A, placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the virally encoded VEGF-E (47). VEGF and PlGF are known to stimulate endothelial cell proliferation, migration and tissue invasion, suggesting that they may also play a role in these processes in EVT cells. This is noteworthy since EVT cells eventually become very endothelial-like when they replace endothelial cells surrounding maternal arteries. There are three known receptors for VEGF; flt-1, KDR, and flt-4, which are tyrosine kinase receptors with 7 transmembrane loops (48). PlGF binds only to the flt-1 receptor, and not the KDR receptor. Since significant expression of VEGF, PlGF and the flt-1 receptor has been demonstrated in various cell-types at the maternofetal interface, many groups have investigated the role of VEGF family in trophoblast differentiation and invasion.

VEGF mRNA is most abundantly produced by decidual macrophages (49), and is also expressed in isolated term CTB cells (50). Immunohistochemical and *in situ* hybridization techniques have localized VEGF in the villous CTB and STB layers (51), although STB VEGF immunoreactivity is weak (52). Using the SGHPL-4 CTB cell line grown on fibrin gel, Lash *et al.* have demonstrated that treatment with exogenous VEGF results in a decrease in trophoblast invasion, but an increase in migration, without affecting proliferation (53). The inability of VEGF to stimulate invasion has also been ascertained by other groups, using slightly different *in vitro* systems. Athanassiades and Lala have used an *in vitro* propagated EVT cell line in Matrigel invasion and motility assays, and found that VEGF had no effect on either migration or invasion (54). In other studies, VEGF has been shown again to have no effect on invasion or motility, but to increase trophoblast proliferation (54). The inconsistency of the effect of VEGF on trophoblast proliferation is most likely due to differences in the type of trophoblast cell used; whether they were freshly isolated or one of the various immortalized trophoblast cell lines currently in use.

PlGF is a homodimeric glycoprotein of which two isoforms exist, PlGF-1 and PlGF-2, the latter having a heparin-binding domain (54). PlGF mRNA is highest in trophoblast cells (55), and first trimester EVT *in vitro* express both mRNA and protein for PlGF (54). Like VEGF, exogenous PlGF added to SGHPL-4 trophoblast cells does not affect invasion capabilities, nor does it affect migration. Athanassiades and Lala have shown a stimulatory effect of PlGF-1 on normal EVT cell proliferation in a concentration-dependent manner. These findings place PlGF amongst a large group of growth factors that promote EVT cell proliferation without influencing their migratory or invasive behaviours. Shore *et al.* have demonstrated that the prominent expression of PlGF in isolated trophoblasts can be down-regulated by 73% in hypoxic conditions (1% O_2), which is somewhat puzzling since it is also known that hypoxia stimulates trophoblast proliferation (50).

Flt-1, the shared receptor for both VEGF and PlGF, is expressed in particularly high amounts by CTBs, including EVT cells of cell columns, as well by villous vascular endothelial cells (53). KDR is also expressed by both STB and EVT trophoblast cells (56). Isolated first trimester EVT cells also express mRNA and protein for flt-1. The presence of these receptors suggest non-endothelial effects of VEGF/PlGF on EVT cells to promote proliferation and invasion. However, much of the flt-1 located in trophoblast cells is believed to be a truncated

form, which can be secreted into the circulation and is thought to act as a VEGF antagonist (57). Therefore, the role of the VEGF/PlGF-flt-1 system in EVT cell function is not completely understood and is currently being studied further.

Insulin-like Growth Factors

Insulin-like growth factors (IGFs) are single-chain polypeptides that are part of the same family as proinsulin. The two major forms are IGF-I and IGF-II, which share 62% sequence homology. Both of these growth factors are synthesized in placenta as early as 9W (58). IGF-I mRNA levels are highest in first trimester, decrease slightly by the second trimester and are lowest at term (59). IGF-I has been immunolocalized to the STB layer, and it is thought that IGF-I secretion by the STB may influence EVT differentiation and invasion (60). Early studies on isolated placental trophoblasts have demonstrated the ability of IGF-I to potentiate EGF-stimulated hPL secretion, thereby implicating IGF-I in trophoblast differentiation (61). In first trimester placental villous explants, Aplin *et al.* have shown that the addition of IGF-I results in an increase in streaming of EVT cells at the edges of outgrowths, indicating that IGF-I stimulates EVT cell migration (62).

IGF-II mRNA is higher in second trimester placenta than first trimester or term. It has been immunolocalized in villous fibroblasts and in both villous and extra-villous trophoblast (63). Only EVT retain mRNA expression of IGF-II throughout gestation (64). The effects of IGF-II on trophoblast proliferation, migration and differentiation have been addressed using both isolated first trimester EVT cells and first trimester villous explant cultures. Treatment of cultured first trimester EVT cells with IGF-II resulted in an increase in both invasion and migration, as assessed by Matrigel assays (63). This study also addressed whether the type 1 IGF receptor was responsible for the effect of IGF-II on enhanced invasion and migration by treating the cells with an antibody to the type 1 receptor. They found that this antibody had no effect on IGF-II effects, suggesting that the type 1 receptor does not mediate IGF-II action on EVT cells. In the villous explant system, exogenous IGF-II, like IGF-I, was found to increase streaming of outgrowth EVT cells, indicating a stimulation of migration. However, when they added an antibody to IGF type 1 receptor, the effect of IGF-II on enhanced streaming was abolished (62). *In vivo*, both STB and CTB are known to express type 1 and type 2 IGF

receptors (65), but as these studies indicate, the role of these receptors in mediating IGF-I and IGF-II signaling is unclear.

IGF actions are also modulated by IGF-binding proteins, of which 10 human isoforms have been identified (63). They play a pivotal role in determining the biological stability of IGF-I and -II, by either enhancing or inhibiting the actions of IGFs at the cellular level. They enhance IGF-mediated biological actions by retarding the degradation and clearance of IGFs or by facilitating the binding of IGF to their receptors (63). One mechanism by which they enhance binding is by interacting with Arg-Gly-Asp (RGD) sites on cell surface integrins, such as $\alpha5\beta1$ integrin, and then recruiting the IGF. STB, intermediate CTB and fetal membranes exhibit weak immunoreactivity for IGFBP-1 at either the 1st TM or term (60). Decidual cells, however, express high levels of IGFBP-1 (66-68), and are likely the source of the high levels of circulating IGFBP-1 in the first trimester. Hamilton *et al.* have examined the effects of IGFBP-1 on the proliferation, migration and invasiveness of cultured human first trimester EVT cells, and found that it increased both invasion and migration, without affecting proliferation (63). Interestingly, when incubated in the presence of an antibody to $\alpha5$ integrin, the stimulatory effects of IGFBP-1 on migration and invasion were abolished, indicating that the effects of IGFBP-1 depend on access to cell surface $\alpha5$ integrin. This is supported by Irwin *et al.*'s findings that an antibody to $\alpha5$ integrin can co-precipitate IGFBP-1 when CTB extracts are incubated with IGFBP-1 (69). Furthermore, in this study the attachment of CTB cells to fibronectin substratum was inhibited by an RGD-containing octapeptide, by antibodies to $\alpha5$ integrin, and by IGFBP-1, supporting the notion that IGFBP-1 binds to the RGD-binding site of $\alpha5$ integrin. However, in invasion assays, this group found that CTB showed limited invasion into endometrial stromal multilayers decidualized *in vitro* and secreting abundant IGFBP-1, but invaded the multilayers when IGFBP-1 was inhibited by insulin. They conclude that it is likely that IGFBP-1 produced by the decidua acts to limit EVT invasion. These differing effects of IGFBP-1 on trophoblast invasion require further study.

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a pleiotropic cytokine that was first characterized by its ability to promote the proliferation of hepatocytes *in vitro* (70). Subsequently, HGF has been found to play a role in cell migration, proliferation and morphogenesis in a number of different

cell types and tissues (71). The receptor for HGF is a tyrosine kinase receptor and is the product of the *c-met* proto-oncogene (72). A potential role for HGF during pregnancy was first suggested in studies demonstrating high levels of HGF mRNA and protein in the human placenta (73). Furthermore, HGF null mutations in mice are lethal to the embryo due to abnormal placental development (74). The site of human placental synthesis of HGF is villous core mesenchymal cells, whereas trophoblast cells express the Met HGF receptor (75). To determine the significance of HGF in trophoblast cell function, Kauma *et al.* treated the ED27 trophoblast cell line with HGF and observed a dose-dependent increase in ED27 invasion (these cells express Met but do not produce HGF) (76). Furthermore, they found a 2-fold increase in 92 kD collagenase activity, which is the matrix-metalloproteinase (MMP) that is thought to be the major regulator of trophoblast invasion. These data suggest that HGF produced and secreted by the villous core may play a role in the regulation of trophoblast invasion *in vivo*. It is possible that as EVT cells migrate further away from the villous core, there would be a lower concentration of HGF present to stimulate trophoblast invasion, which may be a mechanism by which trophoblast invasion is limited.

The ability of HGF to promote trophoblast invasion has also been shown in the human EVT cell line SGHPL-4, wherein treatment with HGF results in a significant stimulation of cell motility in monolayer culture, and invasion into fibrin gels (77). This study also determined the role of nitric oxide (NO) in the HGF-stimulated effects – they found that the NO inhibitor L-NMMA inhibited invasion, motility and cGMP production induced by HGF. Cell motility was also significantly inhibited by the inducible nitric oxide synthase (iNOS)-specific inhibitor 1400W. The data presented in this study demonstrate a direct effect of trophoblast-derived NO synthesis on trophoblast cell function and support the idea that HGF is involved in the regulation of trophoblast invasion through mechanisms that involve the production of NO.

Transforming Growth Factor-β Superfamily

The role of transforming growth factor-β (TGF-β) superfamily members in human placental development and specifically in EVT differentiation and invasion has been studied extensively during the past 10 years, but the precise functions of these cytokines remain unclear and controversial. Overall, it is thought that TGF-β suppresses EVT invasion (78, 79). TGF-β is a polypeptide growth hormone composed of two disulfide-linked monomers, and at least 5

different molecular species are recognized (80). The human placenta produces large quantities of TGF-β1, and it was from this tissue that TGF-β1 was initially purified and characterized (81). It is abundant in the ECM and early gestational decidua (8). Furthermore, Vuckovic *et al.* have detected immunoreactivity for TGF-β in the cytoplasm of STB in both first trimester and term placenta, with most intense staining observed in first trimester trophoblastic syncytial sprouts (82). In a comprehensive examination of term placenta, Schilling *et al.* determined by RT-PCR that mRNA for TGF-β1, -β2 and -β3 are expressed in the term placenta (83). Moreover, they found intense staining for all TGF-β isoforms in the STB layer, chorionic plate, and in cells of EVT columns using IHC.

Graham and Lala have determined that TGF-β is a decidual-derived factor that inhibits trophoblast invasiveness by incubating trophoblast cultures with conditioned media from first trimester decidual cell cultures, which results in an inhibition of trophoblast invasiveness (84). Neutralizing antibodies against TGF-β could prevent the anti-invasive activity of the conditioned media. They also determined that TGF-β is produced in an active form by trophoblasts themselves in culture. Furthermore, they found that TGF-β inhibits proliferation of CTB cells in culture, and induces them to form multinucleated cells. Taken together, this study suggests that the main role of TGF-β in trophoblast biology is to inhibit proliferation and invasion, and perhaps to induce syncytialization.

The role of TGF-β1 in promoting syncytialization has also been investigated by Getsios *et al.* One mediator of the fusion of CTB to syncytium is the novel cadherin subtype, cadherin-11. In this study, TGF-β1 was shown to increase cadherin-11 mRNA and protein expression in primary cultures of EVT cells in a dose-dependent manner (85). Cadherin-11 was immunolocalized to large cellular aggregates and multinucleated cells that formed in response to increasing concentrations of TGF-β1. However, other studies have shown that the syncytialization of CTB cells induced by EGF (as discussed above) is inhibited by TGF-β (86), inferring a role for TGF-β as an anti-syncytialization molecule.

The ascribed role of TGF-β in inhibiting trophoblast invasion is not supported by all studies addressing this topic. Bass *et al.* have found that exogenous TGF-β1 has no effect on either trophoblast cell morphology or the rate of cultured first trimester trophoblast invasion *in vitro* (38). In addition, there has been a report in which the exogenous treatment of first trimester

trophoblast cells with TGF-β has resulted in the induction of the 92-kD collagenase (MMP-9), which, as will be discussed later, is thought to be a major contributor to trophoblast invasiveness.

Caniggia *et al.* have used the placental villous explant system to try to elucidate the role of TGF-β3, activin (another cytokine in the TGF-β superfamily), and follistatin (an activin binding protein) in EVT differentiation and invasion (87). Treatment of explants with activin induced larger outgrowths of EVT cells than those of control explants, and these explants produced more fibronectin, an ECM component produced by EVT cells in cell columns *in vivo*, as well as more hCG and progesterone, two hormones produced mainly by STB. Since the activin receptor ActIIB is also expressed on the STB layer, it is believed that activin acts on the STB layer to induce hCG and progesterone release (80). The increase of these hormones in this study supports this role for activin. Fibronectin production coincides with the overall size of outgrowths; therefore the observed increase in fibronectin synthesis with activin treatment is expected if the outgrowths are larger than control.

The role of TGF-β3 in EVT differentiation is also somewhat controversial. Caniggia *et al.* have implicated this molecule as a key regulator in EVT differentiation, again using the villous explant system (88). *In vivo*, they have found that TGF-β3 mRNA is highly expressed during early placentation (6-8 weeks) when oxygen tension is low, and that expression declines at 10-12 weeks, when oxygen tension increases. However, Lyall *et al.* have used 2 quantitative RT-PCR methods and found no changes in either TGF-β1, β2, or β3 mRNA throughout the first trimester (89). Obviously, the complex signaling events mediating EVT differentiation and invasion are not easy to dissect.

Interleukins

Primarily regarded as mediators of immunologic responses, interleukins (ILs) have also been detected at the maternofetal interface, where they may play an important role in regulating the expression of MMPs, enzymes required for the degradation of maternal decidual extracellular matrix. Transcripts for IL-10, IL-6, TNF-α, and IL-1β are expressed in first trimester (7-9 week) placenta, whereas IL-2 and IL-1α transcripts were very weakly or not at all detected in this study (90). There have been many reports implicating some of these interleukins in MMP induction. Shimonovitz *et al.* have determined that although isolated first trimester trophoblasts constitutively express two species of collagenase type IV (MMP-9 and MMP-2), treatment with

IL-1 further augments MMP-9 secretion in a dose-dependent manner (91). This effect is neutralized by addition of soluble IL-1 receptor, suggesting a receptor-mediated response. Librach *et al.* also treated isolated trophoblast cells with IL-1β, and observed enhanced invasion in Matrigel and an increase in secretion of MMP-9 that required nascent mRNA and protein synthesis (92). IL-6 has also been shown to induce MMPs in purified first trimester CTB cultures. Treatment with IL-6 for 4 days increased the activity, but not the immunoreactivity, of both MMP-2 and MMP-9 (93).

Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is synthesized by a variety cell types, including vascular endothelial cells, monocytes/macrophages, certain neurons, and placental CTBs (94). PDGF signals via homo- or heterodimers (of PDGF-A and PDGF-B) on two receptor types, PDGFRα and PDGFRβ (95). The successive activation of PDGF-B and PDGFRβ genes is thought the play a role in the genesis of choriocarcinoma, the cancer derived from trophoblast cells (96). Furthermore, mice with null mutations in PDGF-B and PDGFRβ display developmental abnormalities in the labyrinthine layer of the placenta, such as dilated embryonic blood vessels and reduced numbers of labyrinth trophoblast cells (94). PDGF is also thought to be involved in the regulation of the proliferative potential of EVT cells in the human placenta (97). Holmgren *et al.* have determined that PDGFRα and PDGFRβ are expressed by human EVT cells at term both *in vivo* and *in vitro* (97), but that while the PDGFRα is located at the cell surface, the β receptor is only detected intracellularly. They have also detected significant amounts of PDGF-B protein production by isolated CTB cells, supporting other studies that have found that levels of PDGF-like activity in the medium of placental explants are measurable in the same range reported in human tumour cells lines producing PDGF (98). When exogenous PDGF-AA or PDGF-BB is added to primary cultures of term CTB, there is a significant increase in DNA synthesis, suggesting that PDGF may be involved in the regulation of EVT proliferation *in vivo* (97). Gurski *et al.* have recently detected PDGF-AA protein in term human placentas as well, which is localized to the endothelial layer of fetal vessels and the STB layer (99). In first trimester placenta, villous trophoblasts do not express PDGF-B nor the PDGF receptors, but invaded EVT have been reported to express both (100), which is somewhat contrary to the proposed role of PDGF in promoting cell proliferation.

Growth Factor Conclusions

The effects of various growth factors on trophoblast cells remain ambiguous at present. A confounding issue in this area of research is that many types of trophoblast culture systems are used. Many groups isolate trophoblast cells from placentas for culture, but these can range in gestational age from first trimester through to term. The ages of the placenta from which the trophoblast cells are isolated likely affects the expression of growth factor receptors in these cells, since *in vivo* immunolocalization studies have demonstrated age-dependent changes in receptor expression. Growth factors may therefore elicit markedly different effects on isolated trophoblast cells from different placentas. Trophoblast cell lines are also used in many studies, derived from either choriocarcinoma cells or immortalized from isolated trophoblast cells. Like most cell lines, these likely express different levels of growth factor receptors and downstream signaling molecules compared to their parental trophoblast cell-type. The intepretation of the results of growth factor treatment on these cells should also be made with caution. Placental villous explant cultures are also used to assess the effects of growth factors on trophoblast biology. These most closely mimic the *in vivo* situation, but the large heterogeneity in the placentas from which villi are isolated could produce differences in growth factor effects. This is especially true for first trimester placentas, since the women from which the samples are collected could be smokers, drug-users, alcohol-users, etc.

Studies of growth factors on trophoblast function, regardless of the trophoblast system used, tend to be limited to two major endpoints: effects on proliferation and effects on invasion. Although there are established methods for evaluating both of these in trophoblast cells, the major effects of some growth factors may not be in affecting either of these phenomena. To more fully understand the role of growth factors in trophoblast biology, other effects could also be evaluated.

1.4.3 Matrix Metalloproteinases/TIMPs

Trophoblast differentiation and invasion into the maternal decidua requires that the trophoblast cells be able to degrade the decidual extra-cellular matrix (ECM). Early *in vitro* studies have demonstrated the capacity of trophoblast cells to degrade ECM components (101), and immunohistochemical and biochemical studies have identified that a number of proteinases,

as well as their activators and inhibitors, are involved in human trophoblast invasion (9). CTBs produce MMPs (102), a group of zinc-dependent endopeptidases that are secreted as inactive proenzymes and are classified into three subfamilies according to their substrate specificities: collagenases, gelatinases and stromelysins (103). The collagenases digest collagen types I, II, III, VII, and X, whereas the two gelatinases digest collagen type IV and denatured collagen (gelatin). The invasive pathway of EVT cells traverses different ECM components, and different MMPs are active to degrade these components accordingly. This is summarized in Figure 1.3.

MMP-1 is a collagenase that degrades interstitial collagens, such as collagens I and III, which are the most abundant collagens in the decidua. An early study by Emonard *et al.* demonstrated that invasive EVT cells express MMP-1, and expression is most prominent once the cells have invaded deep into the decidua and are located intravascularly (104). More recently, Vettraino *et al.* detected MMP-1 protein in second and third trimester villous CTB and STB, but they did not indicate positive staining in deeply invaded EVT cells, although their samples may not have contained this cell population, since specialized sampling methods are required to obtain the placental bed (which includes portions of the maternal decidua that would contain the deeply invaded EVT cells) (105).

MMP-2 (72 kDa-type IV collagenase or gelatinase A) expression has been demonstrated in EVT cells by immunohistochemistry and *in situ* hybridization (106-108). According to *in situ* hybridization studies to localize MMP-2 mRNA, this proteinase is not expressed in proliferating EVT, but only in the invasive phenotype throughout all stages of pregnancy. Huppertz *et al.* have confirmed this at the protein level by the presence of strong immunostaining for MMP-2 and MMP-9 in the distal, invasive part of cell columns (109). *In vitro* studies have also ascribed a role for MMP-2 during the process in which villous CTBs break through the basal lamina of villi to become extra-villous, in the earliest stages of column formation (87).

MMP-3 (stromelysin-1) has also been implicated in the differentiation of EVT in anchoring cell columns. This proteinase degrades fibronectin, laminin, various collagens and core proteins of proteoglycans. Studies by Kaufmann *et al.* have detected expression of MMP-3 by immunohistochemistry in the late invasive EVT phenotype, with immunoreactivity being extracellular and linked to fibronectin fibrils in the ECM (9).

MMP-7 (matrilysin) is another proteinase with a broad range of substrate specificities, and is present and active during trophoblast invasion according to Vettraino *et al.* (105).

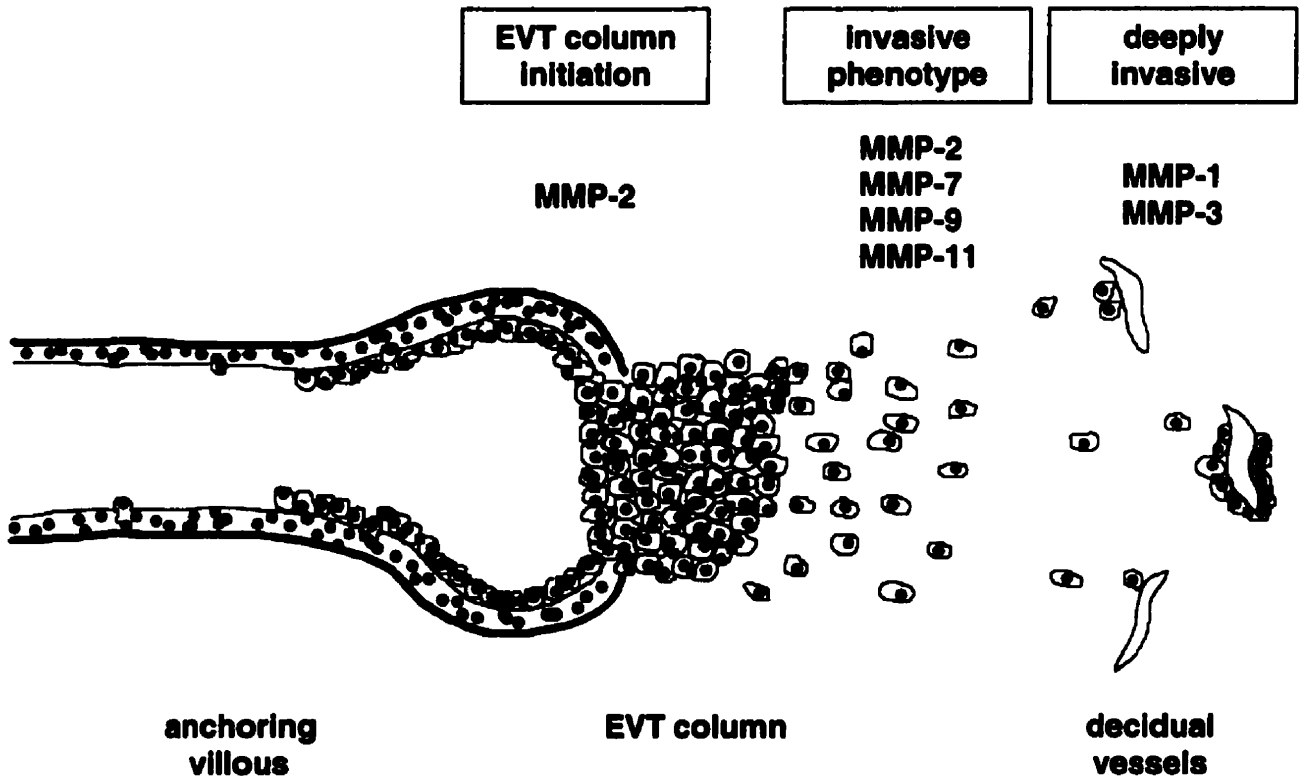

Figure 1.3. Matrix-metalloproteinases in EVT differentiation and invasion. MMP activities change along the invasive pathway. MMP-2 is required for column initiation, MMP-2, -7, -9, and -11 are active during trophoblast invasion, and MMP-1 and -3 are active in late invasive EVT cells deep in the decidua.

MMP-9 (92 kD-type IV collagenase, gelatinase B) is present at both mRNA and protein levels in the proximal, proliferating layers of EVT cell columns, and then expression is decreased during differentiation into the early invasive phase. MMP-9 expression is then upregulated once again during late EVT invasion (9). The *in vitro* invasive potential of differentiated EVT cells seems to be mediated by MMP-9, since antibodies against MMP-9 completely inhibit trophoblast invasion *in vitro* (110).

MMP-11, a laminin and collagen IV-degrading MMP is expressed only by the invasive EVT phenotype, and its expression decreases with advancing gestation (111).

The activity of MMPs by EVT cells along the invasive pathway parallels the ECM environment in which the cells are located. The proliferating CTB cells at the very base of the column rest on a basal lamina composed of collagen IV, laminin, and heparan sulfate (9). According to some studies, the activity of MMP-2 may be required to degrade the collagen IV of this environment in order to facilitate the CTB cells to leave the basal lamina and begin migrating away from the villous tip (87). The typical ECM of the invasive stages of EVT cells is composed of a patchy mosaic of collagen IV, laminin, fibronectin, oncofetal fibronectin, and heparan sulfate (9). The most prominent and important MMP during this phase is MMP-9, which also degrades collagen IV. However, as invasion continues and more fibronectins, proteoglycans and interstitial collagens are encountered, MMP-1 and MMP-3 are also active (9).

MMP activity is modulated by a group of tissue inhibitors of metalloproteinases (TIMPs). So far in the human placenta, two of these inhibitors have been immunolocalized: TIMP1, which binds to the active forms of all MMPs, except for MMP-9, to which it binds to the inactive proform (112), and TIMP2, which preferentially interacts with and inhibits MMP-2 (113). Both of these TIMPs are expressed by decidual cells (108), but not by CTB cells in culture (114). In the decidua, TIMP1 activity increases towards term, whereas TIMP2 expression remains constant throughout pregnancy (108). This upregulation of TIMP1 throughout pregnancy and the constant presence of TIMP2 may limit EVT cell invasion into the decidua by inhibiting the MMPs that are so integral to their invasion. This is supported by *in vitro* studies wherein exogenous TIMP1 or TIMP2 added to human CTB cultures can completely inhibit invasion (9).

1.4.4 Integrins/Cadherins

Just as MMPs are spatially and temporally regulated during trophoblast differentiation along the EVT invasive pathway, so too are cell-cell adhesion molecules and the receptors for the various ECM components that invading trophoblasts encounter. The changes in cadherin and integrin expression have been mapped by Damsky and Fisher, and are summarized in Figure 1.4. (115). Integrins constitute a large superfamily of heterodimeric, transmembrane adhesion receptors that transduce signals from ECM components. There are many known αβ subunit combinations for a single integrin receptor, and redundancies exist in their ligand preferences (114). The complexity of understanding integrin signaling is enhanced by the existence of alternative splicing of cytoplasmic domains of integrin subunits which would alter intracellular signaling cascades, and the existence of multiple affinity states of many integrins for their specific ligands. Cadherins are a family of cell-surface glycoproteins that function in promoting calcium-dependent cell-cell adhesion, and also serve at the transmembrane components of cell-cell adherens junctions (116). The classical cadherins are E-, N-, and P-cadherin, which are expressed in a cell-, tissue-, and development-specific manner. E-cadherin is known to be the major cadherin in polarized epithelial cells.

Damsky *et al.* have undertaken extensive histologic studies to determine the dynamic localization of integrins and cadherins along the EVT invasive pathway (115). CTB stem cells within villi express αvβ5 integrin, and at site of column initiation in the first trimester αvβ5 staining extends into the first 2-3 layers of the column (117). Furthermore, αvβ6 integrin is located only in villous CTB that are at sites of column initiation. The early proliferative CTB cells in the proximal column also express high levels of E-cadherin and stain strongly for α6β4 integrin. The first step of differentiation, when EVT cells stop proliferating within the cell columns, is accompanied by the upregulation of the α5β1 fibronectin receptor, coincident with entrance into a fibronectin-rich ECM. Further along the differentiation pathway, when individual cells leave the cell columns and invade the uterine wall, EVT cells express the α1β1 collagen/laminin receptor, as well as continuing to express the α5β1 fibronectin receptor. The αvβ5, αvβ6 and α6β4 integrins are no longer detected and E-cadherin staining is weak and discontinuous (115). This integrin switching suggests that the modulation of adhesion receptor phenotype is functionally important for the development of the invasive EVT phenotype. Using *in vitro* differentiation studies of isolated first trimester CTBs, Damsky *et al.* have also

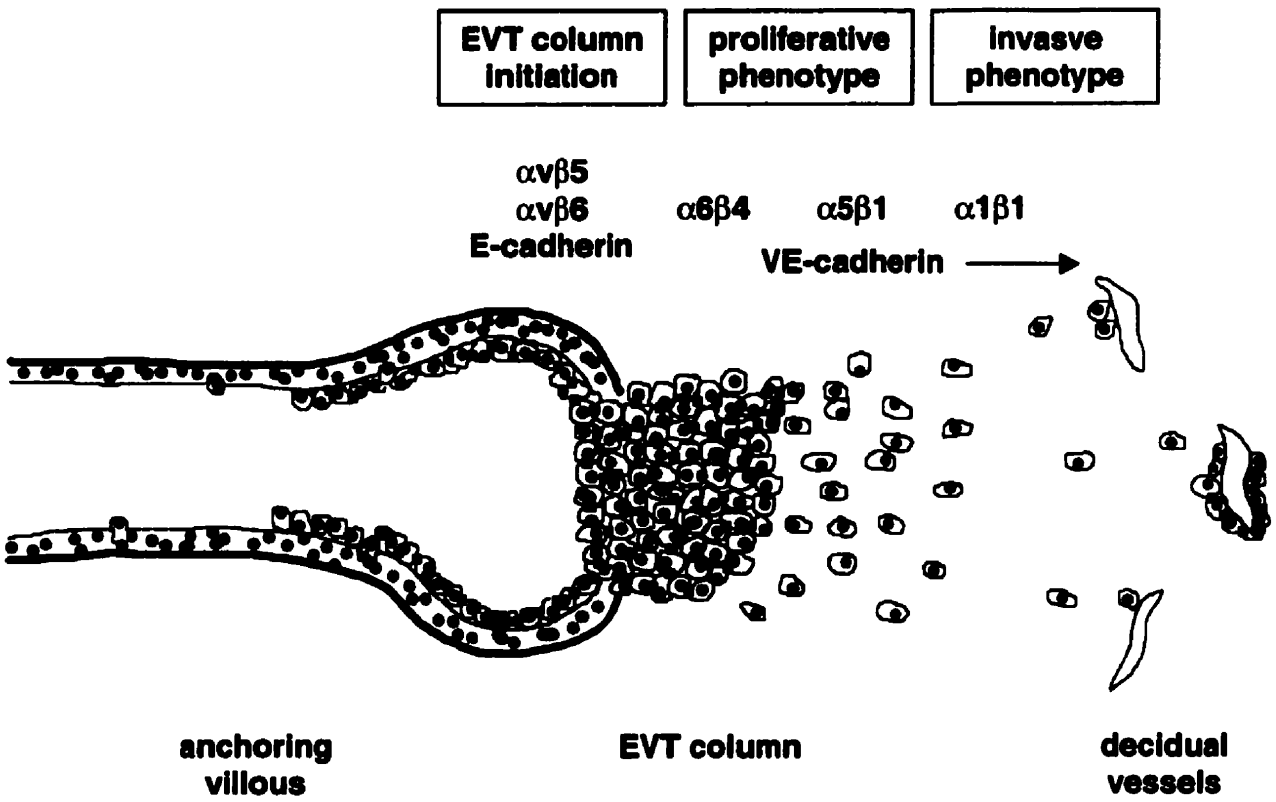

Figure 1.4. Integrins and cadherins in EVT differentiation and invasion. CTB cells within villi express αvβ5 and αvβ6 integrins. In the first 2-3 layers of an EVT column, αvβ5 is expressed, as well as E-cadherin. In the remainder of the proliferating column, α6β4 integrin is expressed. Post-mitotic EVT cells express α5β1, and fully invasive EVT express α1β1. When EVT cells breach maternal vessels, they upregulate VE-cadherin expression.

performed antibody-perturbation experiments to determine the functional consequences of integrin switching in EVT differentiation (118). Their data suggest an invasion-promoting role for $\alpha 1\beta 1$ and an invasion-restraining role for $\alpha 5\beta 1$, and they speculate that EVTs might regulate the depth of their invasion in part by titrating the net effects of these opposing integrin actions. Furthermore, the upregulation of $\alpha 1\beta 1$ in individual invasive EVT cells deep in the maternal decidua is of interest since endothelial cells surrounding maternal spiral arteries also express this integrin. Zhou and Damsky propose a model in which invading EVT cells adopt a vascular phenotype in order to appropriately function as endothelial-like trophoblasts when they breach maternal spiral arteries (117).

The down-regulation of E-cadherin early in EVT differentiation may also have a functionally significant role. E-cadherin is known to be down-regulated in many tumour cells of epithelial origin, strongly correlating with tumour invasiveness (119). Therefore, the observed decrease of E-cadherin in EVT differentiation may be permissive for invasion. Zhou *et al.* have also immunolocalized VE-cadherin, the endothelial adhesion molecule, in the early human placenta (117). Anti-VE-cadherin antibodies stained endothelium of villous fetal blood vessels as well as EVT cells in distal cell columns and in the decidua, where E-cadherin expression is low. Furthermore, maternal decidual endothelial cells lining maternal vessels expressed only VE-cadherin, and not E-cadherin. Therefore, as the EVT cells approach their ultimate destination, maternal spiral arteries, they adopt a membrane phenotype that approximates the endogenous endothelial cells that they seek to replace. In experiments using antibodies to functionally block cadherins in freshly isolated CTB cells seeded on Matrigel-coated Transwell inserts, Zhou *et al.* found that blocking E-cadherin function resulted in a 3-fold increase in trophoblast invasiveness, whereas blocking VE-cadherin caused a 60% reduction in invasiveness. Therefore, just as a switch in integrin expression appears to regulate invasiveness, so too might a switch in cadherin expression.

Another membrane protein with relevance to EVT differentiation and this thesis project is HLA-G (human leukocyte antigen-G), a non-classical major histocompatibility complex (MHC) class I molecule. MHC class I molecules are membrane-bound glycoproteins that are ubiquitously expressed in almost all tissues, the exception being the central nervous system (120). These polymorphic molecules have long been known to be responsible for allograft rejection, since MHC class I molecules are different amongst individuals (121). Since the

placenta is of fetal origin, composed of both maternal and paternal antigens, most human trophoblast cells are unique in that they do not express any MHC class I molecules (122). EVT cells of cell columns express the non-classical HLA-G, and the precise functional significance of this is still being elucidated (123). For the purpose of this thesis, a specific antibody against HLA-G was very useful as a marker for EVT cells in cell columns.

1.4.5 Changes in Oxygen Tension

A final important mediator of EVT differentiation is oxygen tension. As discussed by Genbachev *et al.* in their 1997 publication in *Science*, oxygen tension in the placenta changes throughout pregnancy, and more importantly there is a gradient of oxygen that EVT cells encounter along the invasive pathway (124). At 9 weeks gestation, the mean oxygen pressures of the intervillous space and within the endometrium are estimated to be around 18 mmHg and 40 mmHg respectively. Therefore, prior to 9 weeks gestation placental villi are located in a low oxygen environment (18 mmHg), but as EVT cells invade into the endometrium they encounter a gradient increase in oxygen tension (to 40 mmHg). After 9 weeks, EVT cells breach maternal spiral arteries, thus increasing blood flow and hence oxygenation of intervillous space blood, and the partial pressure of oxygen rises to approximately 60 mmHg in the intervillous space for the remainder of pregnancy (125).

Genbachev *et al.* have demonstrated the difference between low oxygen (2% O_2 or 14 mmHg) and standard oxygen (20% O_2 or 98 mmHg) conditions on explants of first trimester human placental villi by examining some markers of trophoblast differentiation (124). First trimester villous explants grown under both low and standard culture conditions will grow outgrowths of EVT cells, analogous to cell columns *in vivo*. Under 20% O_2 conditions, the EVT cells in these outgrowths are not proliferative, as determined by BrdU incorporation studies, whereas when the explants are maintained in low oxygen, the nuclei of many of the CTBs in the outgrowths incorporate BrdU, indicating that they are proliferative. Furthermore, the outgrowths protruding from villous tips grown in low oxygen are larger than those cultured under standard oxygen conditions (mean of 1476 cells compared to 516 cells). By examining the expression of proteins that regulate the cell cycle in these explants, they determined that hypoxia stimulates EVT cells to enter mitosis. As for their invasive capabilities, outgrowths of explants cultured in low oxygen do not express invasive markers such as $\alpha 1$ integrin and human placental lactogen,

whereas under 20% O_2 conditions these markers are up-regulated. The low oxygen explants do however maintain expression of HLA-G and α5 integrin, indicating that these markers are not oxygen-dependent. The importance of the intrinsic proliferative capacity of CTB cells in the low oxygen environment of the early human placenta (prior to 9 weeks) is evident when considering the growth of the placenta compared to that of the embryo, which is subject to similar oxygen conditions. During this early period, the mass of the placenta increases much more rapidly than that of the embryo proper – therefore the intrinsic stimulation of mitosis in CTBs by low oxygen tension allows the placenta to rapidly develop in order to function early enough to maintain embryonic growth. The gradient in oxygen tension as EVT cells invade may be pivotal to their differentiation process, since the higher oxygen tension would limit their proliferation and induce differentiation into a more invasive phenotype. This might also explain why EVT cells preferentially invade maternal spiral arteries, and not veins, since the oxygen tension is much higher in spiral arteries than in veins.

The mechanisms by which a cell senses oxygen tension are known to involve a crucial transcription factor, hypoxia-inducible factor 1 (HIF-1) (126). HIF-1 is an αβ heterodimer, both subunits belonging to the PAS family of bHLH transcription factors. The β subunit is identical to the aryl hydrocarbon nuclear translocator (ARNT) that also serves as a heterodimeric partner with the aryl hydrocarbon receptor (127). The α subunit, on the other hand, has the sole function of mediating responses to oxygen. Like the other bHLH transcription factors already discussed, the N-terminal basic domains of HIF-1 dimers bind to DNA at hypoxia-response element (HRE) consensus sequences to promote transcriptional activity, and downstream PAS domains are required for dimerization and target gene specificity (126). Altering oxygen tension has no significant effect on steady-state levels of either HIF-1α mRNA or HIF-1β mRNA (128), but at the protein level, both are remarkably unstable in cells exposed to oxygen (129). Huang *et al.* have demonstrated that the degradation of HIF-1α protein in standard oxygen conditions (20% O_2) is controlled by an oxygen-dependent degradation (ODD) domain within HIF-1α, and that the degradation is catabolized through the ubiquitin-proteosome pathway (126). An important protein mediating HIF proteosome degradation is the von Hippel-Lindau protein (pVHL), which shuttles back and forth between the nucleus and the cytoplasm, forming multimeric complexes that are required for ubiquitination and hence degradation of HIF-1α and HIF-1β protein in the presence of oxygen (130). The classical genes that are regulated by HIF-1 include the

hematopoietic growth factor erythropoietin, angiogenic growth factors such as VEGF, and genes involved in glucose transport and metabolism such as the glucose transporter-1 (GLUT1) (131-133).

Isolated trophoblasts from first trimester placenta express HIF-1α, HIF-2α, and HIF-1β (28). Furthermore, analysis of their transcript levels throughout the first trimester in the human placenta has been performed by several groups with conflicting results. By Northern blot, Rajakumar *et al,* have revealed that HIF-2α mRNA increases significantly with gestational age, whereas HIF-1α remains unchanged (125). Caniggia *et al.* found by *in situ* hybridization and low-cycle PCR followed by Southern blot that HIF-1α mRNA decreases at 9 weeks, the time at which oxygen tension increases in the intervillous space (134). At the protein level, Rajakumar *et al.* have determined that both HIF-1α and HIF-2α increase with gestational age throughout the first trimester, and interestingly, remain detectable in the second and third trimesters, despite the increase in intervillous space oxygenation. This paradox is addressed in a recent publication by Genbachev *et al.*, who have also found that both HIF-1α and HIF-2α protein is maintained into the second trimester of pregnancy (135). They speculate that the relatively hypoxic environment of the decidua throughout the second trimester is sufficient to maintain the expression of HIF-1α and HIF-2α. Immunolocalization studies demonstrate that HIF-1α is detected in few CTB cells and expression patterns do not change during differentiation, suggesting that HIF-1α does not play a role in trophoblast differentiation/invasion. On the other hand, HIF-2α is expressed in villous stem CTBs, CTBs at sites of column initiation, and those in the proximal column, but expression is mostly cytoplasmic. Relatively more intense nuclear staining is observed in EVT cells in distal columns and the majority of EVT cells that have invaded the uterine wall, in both first trimester and early-second trimester samples. This study also investigated the expression pattern of pVHL in first and second trimester placenta, and found expression in villous trophoblast stem cells, and in EVT cells at sites of column initiation and in proximal columns. Since pVHL functions to target HIF-1α and HIF-1β cytoplasmically for degradation, Genbachev *et al.* suggest that in the proliferative EVT phenotype of the proximal column, pVHL co-expressed with HIF-2α sequesters HIF-2α in the cytoplasm, where it can not act as a transcriptional activator. However, when pVHL expression decreases in the more differentiated distal column, HIF-2α protein is able to act in the nucleus as a transcription factor, potentially

activating transcription of angiopoietic genes such as VEGF, allowing them to assume a more vascular phenotype.

In villous explants cultured in either 2% O_2 or 20% O_2, Rajakumar *et al.* found that none of the HIF transcription factors were regulated at the mRNA level, but both HIF-1α and -2α proteins were induced at 2% O_2. Genbachev *et al.* have performed similar experiments with placental villous explants cultured at 2% O_2, and performed Western blot analysis of HIF-1α, HIF-2α and pVHL proteins, with samples containing only column EVT cells (135). They found an upregulation of pVHL and HIF-2α under low oxygen conditions, but no change in HIF-1α protein. Together with their immunostaining data for pVHL and HIF-2α, these data suggest that in the proximal trophoblast column, where oxygen tension is the lowest, pVHL and HIF-2α are expressed but pVHL causes HIF-2α sequestration in the cytoplasm. When oxygen tension increases in the distal column, pVHL is down-regulated, but HIF-2α may not be as oxygen sensitive, and therefore expression persists within the decidua in the absence of pVHL, so that it can now act as a transcriptional activator (diagrammed in Figure 1.5).

Caniggia *et al.* propose that the effects of low oxygen on human trophoblasts are mediated through the transcriptional activation of TGF-β3 by HIF-1α (134). Using villous explant cultures, they have shown that HIF-1α mRNA is upregulated in 3% O_2, contrary to Rajakumar *et al.* and Genbachev *et al.*, and that TGF-β3 is similarly upregulated in these low oxygen conditions. Furthermore, addition of anti-sense oligonucleotides to HIF-1α mRNA to 'knock-down' protein expression in explant culture resulted in a decrease in TGF-β3 expression and a change towards a more invasive phenotype of villous outgrowths. Specifically, anti-sense HIF-1α treatment resulted in the down-regulation of EVT proliferation, MMP-2 activity and α5 integrin expression, and an up-regulation of MMP-9 activity and α1 integrin expression. Although this study contains extensive data supporting their hypothesis, the fact that HIF-1α does not appear to play any role in EVT differentiation *in vivo* (135) suggests that there is a need for further research to more fully understand the role of HIF-1α in trophoblast differentiation.

Hypoxia has also been shown to induce the expression of many cytokines, including TNF-α, IL-1α, and IL-1β (136). As will be discussed in the following section, these cytokines are also known to be upregulated in pre-eclampsia, a placenta-related disorder of pregnancy clinically diagnosed by the onset of maternal hypertension and proteinuria in the third trimester

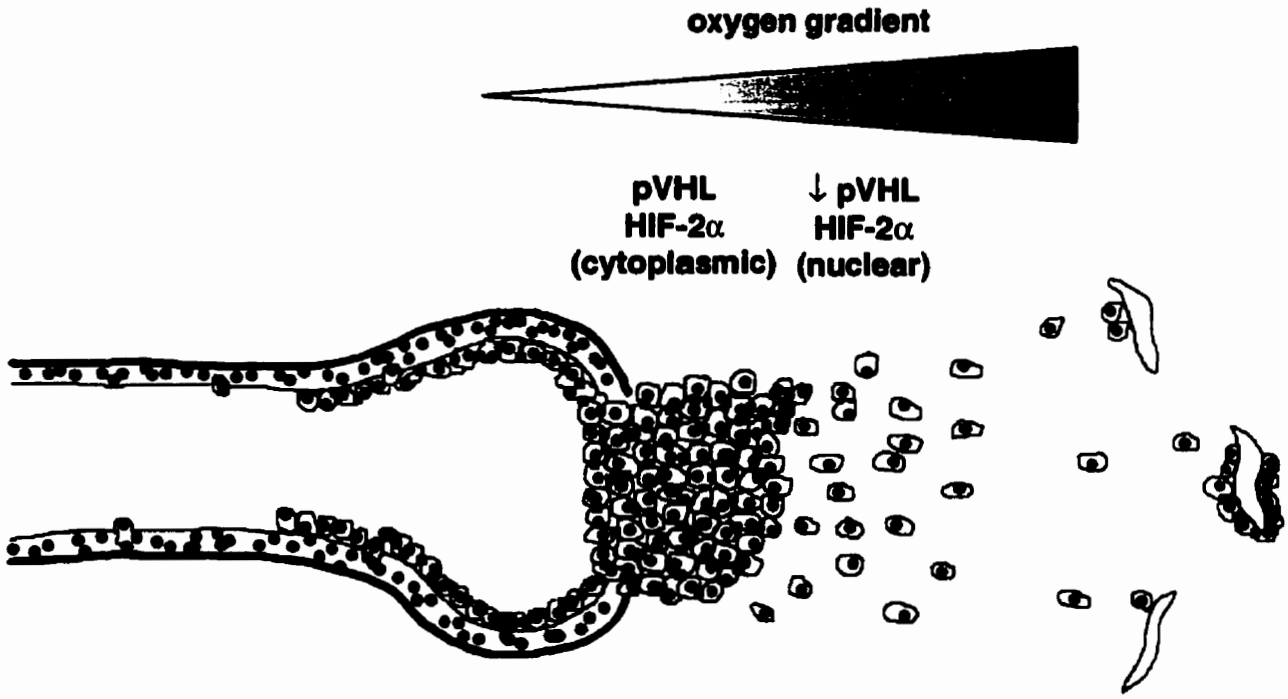

Figure 1.5. Model of the role of hypoxia-inducible transcription factor-2α in EVT differentiation and invasion. In the proximal EVT column, where oxygen tension is the lowest, pVHL and HIF-2α are expressed, and pVHL sequesters HIF-2α in the cytoplasm. When oxygen tension increases in the distal column, pVHL is down-regulated, but HIF-2α expression persists. The lack of pVHL allows nuclear translocation of HIF-2α, where it can act as a transcription factor.

of pregnancy. Although the etiology of this disease is uncertain, it is widely accepted that a defect in placental trophoblast remodeling of maternal spiral arteries contributes to inadequate perfusion of the placenta, causing relative hypoxia. Therefore, just as the low oxygen tension early in pregnancy is integral for EVT cell proliferation, the increase in oxygen tension later in pregnancy is required for proper placental function.

1.5 Abnormal Trophoblast Differentiation/Invasion

Extra-villous trophoblast differentiation and invasion is known to be abnormal in preeclampsia, a disease of pregnancy affecting 7-10% of women and resulting in significant maternal and fetal morbidity and mortality (137). It is currently thought that in preeclampsia, EVT cells inadequately remodel maternal spiral arteries, which leads to a relatively hypoxic placenta for the remainder of pregnancy. The placenta then produces a number of factors that affect the maternal systemic vasculature, leading to uncontrollable maternal hypertension, edema, and proteinuria in the third trimester (138). The symptoms cease upon delivery of the placenta, but this often requires premature delivery by induction or Caesarian section (139).

Since there is no cure for preeclampsia except delivery of the placenta, much research has been devoted to understanding the etiology of this disease, in order to identify potential targets for drug intervention. The hallmark of EVT cells in a preeclamptic placenta is that they do not fully differentiate to the invasive phenotype: they do not express α1 integrin or VE-cadherin, they produce limited MMP-9, and they express little or no HLA-G (140). The lack of HLA-G on invading EVT cells in preeclampsia could target them for lysis by maternal natural killer cells, thus preventing them from adequately remodeling maternal arteries (141). Examination of the placental bed from preeclamptic pregnancies reveals that although EVT cells invade into the decidua, they do not invade the muscle and endothelial layers of maternal arteries, and these vessels remain constricted (141). The absence of α1 integrin and VE-cadherin by EVT in preeclampsia, which are markers of the highly differentiated 'vascular' EVT, has led Fisher *et al.* to postulate that the inability of EVT to mimic a vascular phenotype prevents them from replacing vascular cells lining maternal arteries in preeclampsia (142).

Cytokines and growth factors have also been implicated in preeclampsia. The plasma levels of pro-inflammatory cytokines are elevated in preeclampsia, such as TNF-α and IL-1β, both of which are known to cause endothelial cell dysfunction, a major maternal perturbation in

preeclampsia (143). As mentioned earlier, when normal human placental explant tissues are maintained in reduced oxygen conditions, many pro-inflammatory cytokines are stimulated, including TNF-α, IL-1α, and IL-1β (136). The same correlation has been made with leptin: in preeclamptic pregnancies plasma levels of leptin are significantly elevated compared with gestational age- and body mass index-matched normal pregnant women (144). Plasma leptin levels in preeclamptic women are reduced soon after the placental delivery to those expected for their body mass indices. This study has also shown that a trophoblast cell line exposed to hypoxic (5% O_2) conditions secretes significantly more leptin than when cultured under standard conditions. Kauma *et al.* have also demonstrated that villous explants from preeclamptic placentas produce 25% less hepatocyte growth factor (HGF) compared to normal control villous explants, and suggest that this may contribute to the inadequate trophoblast invasion associated with preeclampsia (76).

TGF-β3 has also been implicated in the pathogenesis of preeclampsia. Caniggia *et al.* propose that a dramatic increase of TGF-β3 mRNA and protein in preeclamptic pregnancies may inhibit normal EVT differentiation to the fully invasive phenotype (88). They report that if explants from preeclamptic placentas are cultured in the presence of anti-sense oligonucleotides to TGF-β3, large outgrowths emerge from villous tips, and MMP-9 activity is upregulated. However, Lyall *et al.* have also examined the expression patterns of all TGF-β isoforms in normal versus preeclamptic placentas, and found no difference in TGF-β3 protein levels by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry between the groups (145). Therefore, the role for TGF-β3 in preeclampsia remains unclear.

Choriocarcinoma is another important clinical manifestation of improper trophoblast differentiation and invasion. This malignancy is unique in that it is derived from trophoblasts, which normally behave in a manner that is interpreted as 'malignant-like.' First, trophoblasts normally show controlled invasion, limited to the decidua and vascular portions of the outer myometrium. Secondly, it is estimated that 100,000 trophoblast cells are deported to the maternal circulation daily, and these are commonly identified in the pulmonary circulation of pregnant women (146). These cells, although analogous to metastases of cancer, do not produce disease. In the case of choriocarcinoma, the complex mechanisms that control the invasion of trophoblast cells are altered and the trophoblast continues to invade and grow without limit, eventually metastasizing and ultimately leading to death (147). Choriocarcinoma is therefore

unique in that it represents a malignant transformation of a tissue that inherently has 'invasive' and 'metastatic' properties. It is also the only tumor which contains DNA foreign to the host, as it is derived from trophoblast cells which contain paternal genetic material. Thus, choriocarcinoma is a complex neoplasm, and to study it, one must study and understand graft rejection, immunologic mechanisms and a multitude of genetic concepts in addition to the mechanisms of trophoblast invasion.

There are other conditions of trophoblast over-invasion, such as placenta accreta, in which trophoblast invades beyond its normal scope, penetrating deeply into the myometrium and even the adjacent serosa (148). The main complication of placenta accreta is hemorrhage at the time of childbirth, which often necessitates hysterectomy (149). The mechanisms underlying this phenomenon remain to be elucidated.

Gap Junctions

Having reviewed human trophoblast differentiation, the remainder of this chapter will focus on gap junctions and their putative role in human trophoblast differentiation.

1.6 Introduction

Gap junctional intercellular communication (GJIC) can mediate cell proliferation, differentiation, and invasion, which are required for many developmental processes and adult tissue homeostasis. Since early human placental development requires trophoblast proliferation, differentiation and invasion, we sought to elucidate the role of gap junctions during human trophoblast differentiation.

1.6.1 Gap junction/connexin protein structure

Gap junctions were first identified as intercellular channels by Makowski *et al.*, who demonstrated that gap junction channels possess a unique structure, spanning the plasma membranes of two adjacent cells (Figure 1.6, A) (150). Each cell contributes one half of the channel (each hemi-channel is called a connexon), which is comprised of 6 connexin (Cx) subunits (Figure 1.6, B) arranged around a central pore. The hydrophilic pore of gap junction channels allows the regulated transfer of ions and small molecules of up to 1.5 kDa between

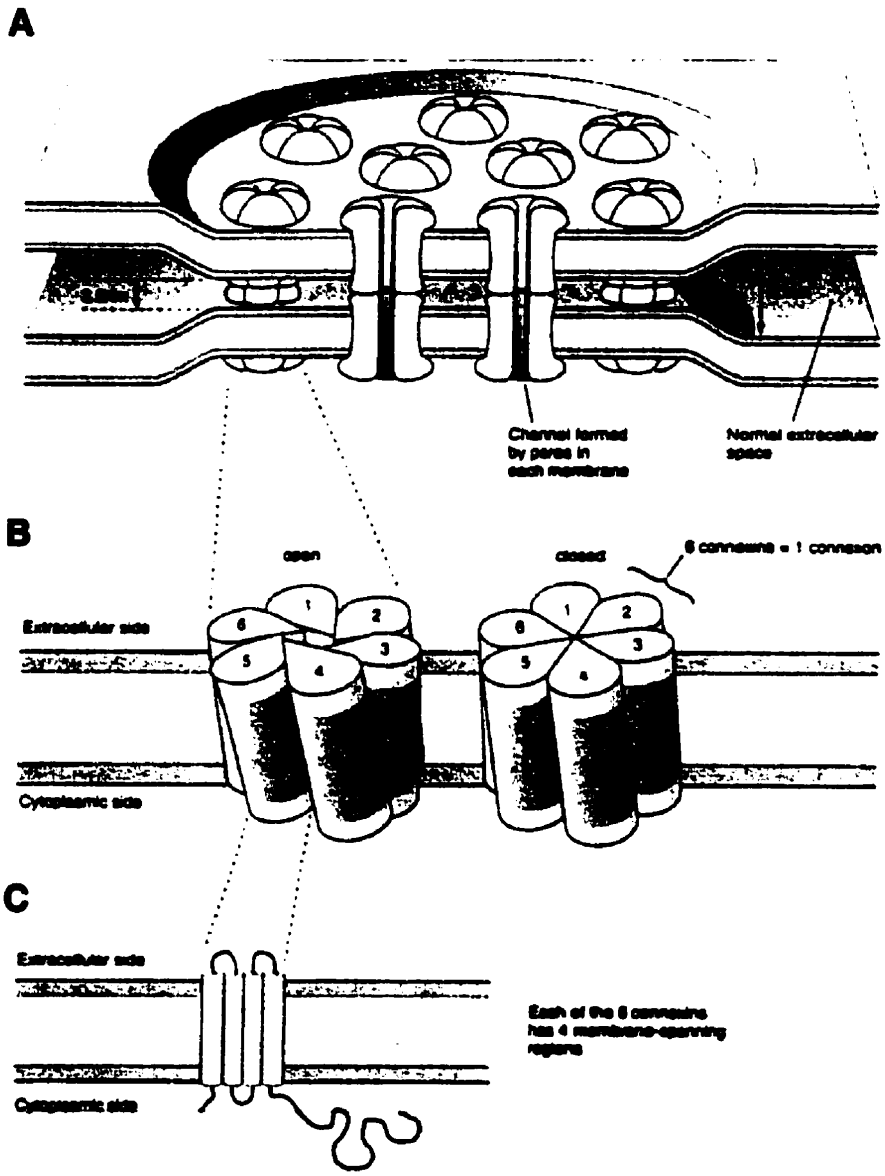

Figure 1.6. Gap junction structure. Gap junctional plaques (A) are composed of a hundred to many thousand gap junctions. A gap junction hemi-channel is composed of 6 connexin subunits to form a connexon (B). Connexins are integral membrane proteins with 4 membrane-spanning α-helices, 2 extracellular loops, a short mid-protein intracellular loop, and a variable C-terminal cytoplasmic domain (C). (Adapted from Bruzzone *et al.*, 1996)

adjacent cells, including cyclic nucleotides (cAMP and cGMP), inositol phosphates, and Ca^{2+} (151). Both the N-terminus and C-terminus of connexin proteins are cytoplasmic, the remainder of the protein is comprised of 4 membrane-spanning α-helices, two short extracellular loops that are thought to regulate connexon-connexon interactions, and a short mid-protein intracellular loop, which is thought to be involved in pH gating of certain connexins (Figure 1.6, C). A high-resolution three-dimensional crystallography structure of a recombinant gap junction channel resolved to 7.5 Å has recently been solved, demonstrating the presence of 24 α-helices per connexon, corresponding to the 4 membrane-spanning regions of each of the 6 connexin subunits, arranged around a central pore (152, 153). Gap junctions aggregate as tightly packed plaques of connexons on cell membranes. Typically, between less than a hundred to many thousand individual channels are combined into a single plaque that can extend from less than a hundred nanometers to several micrometers in diameter (154).

The first connexin was cloned from rat liver by Paul *et al.* in 1986 (155), and since then many other members have been added to this family of proteins. Current nomenclature distinguishes connexins on the basis of the molecular mass of the protein. For example, the 43-kD protein first identified in myocardial gap junctions is termed Cx43. However, Cx43 is also known as α1 connexin, since an alternative nomenclature system exists in which connexins are subclassed into either α or β types, but the specific functional and/or structural criteria for distinguishing these classes have not been defined. The connexin family consists, in rodents, of at least 16 members, with homologues in many vertebrate species. In humans, 11 connexins have been identified, which are summarized on Table 1.1. In general, the predicted transmembrane segments, extracellular loops and the short N-terminal cytoplasmic portion are well conserved amongst connexin family members, but the major C-terminal cytoplasmic domains are unique in both sequence and length (156, 157). Connexins demonstrate tissue-specific expression, and expression is also temporally regulated during development. All adult tissues express at least one connexin that forms functional gap junctions, with the exception of skeletal muscle (151). Some connexons are comprised of different connexin subunits; for example, Cx32 and Cx26 can form such a **heteromeric** connexon when coinfected into Sf9 insect cells (158, 159). Since Cx26 and Cx32 are frequently expressed in the same cell type, it is highly likely that they can also form heteromeric connexons *in vivo*. Direct evidence for heteromeric connexons *in vivo* has been published by Jiang and Goodenough, in which it was

Table I.1. Human Connexins and their Tissue Expressions (adapted from Bruzzone *et al.*, 1996 and Novartis Foundation Symposium on Gap Junctions, 1999).

[illegible]	alveolar cells of lactating mammary gland, intestine, keratinocytes, pancreatic acinar cells, liver
[illegible]	skin
[illegible]	kidney, skin
[illegible]	keratinocytes, kidney
[illegible]	alveolar cells of lactating mammary gland, hepatocytes, neurons, oligodendrocytes, pancreatic acinar cells, proximal kidney tubules, Schwann cells, thyroid follicular cells
[illegible]	cortical neuroblasts, endothelium, heart, keratinocytes, stomach, testes
[illegible]	conductive myocardium, endothelium
[illegible]	astrocytes, cardiac and smooth muscle, endothelium, fibroblasts, keratinocytes, lens and corneal epithelium, leukocytes, marcophages, myoepithelial cells of mammary gland, osteocytes, ovarian granulosa, pancreatic β-cells, Sertoli cells, thyroid follicular cells, myometrium
[illegible]	embryonic tissues (brain, heart, intestine, kidney, lung), blood vessel smooth muscle, adult brain, heart, intestine, kidney, lung, retina, skin
[illegible]	lens fibers, Schwann cells
[illegible]	lens fibers, corneal epithelium

demonstrated that Cx45.6 and Cx56 expressed by chick lens fiber cells localize within the same clusters of channels (160). Another consideration is that a connexon on one cell membrane composed of a certain connexin species may be able to interact with a connexon on an opposing cell membrane composed of a different connexin (**heterotypic** channel). There has been a systematic analysis of the ability of adjacent cells expressing different connexins to communicate, and it appears that some connexins are very selective in docking partners, whereas others non-selective. Cx31, for example, will only form homotypic channels with other Cx31-containing connexons, whereas Cx46 interacts well with all connexins tested except Cx40 and Cx31 (161). Cx40 is also highly restricted in its ability to make heterotypic channels, functionally interacting with Cx37, but failing to do so when paired with Cx32, Cx43, Cx46 and Cx50 (162).

1.6.2 Connexin gene structure

The overall genomic organization of connexin genes appears to be rather simple, consisting of two exons, with most connexins having the complete coding region on exon2 and the 5'-untranslated region divided by an intron over exons 1 and 2 (163). However, a new subgroup has recently been defined of which the gene structure is characterized by the presence of an intron within the coding region (164). The characterization of transcriptional regulatory elements in connexin promoters has only just begun. The Lye lab is currently investigating transcriptional regulation of the Cx43 promoter, which is known to contain a TATA box, AP-1 site, AP-2 site, and a series of half-palindromic estrogen response elements. A recent report by Bierhuizen *et al.* describes progress towards understanding the transcriptional regulation of the Cx40 promoter (165). They have constructed luciferase reporter constructs containing various lengths of 5'-upstream sequences of the rat Cx40 gene, as well as constructs with site-directed mutations in this sequence. They have localized the promoter region of Cx40 and determined that Sp1 and Sp3 transcription factors bind to consensus sites in this region by electro-mobility shift assay. Further analysis is obviously required to begin to understand tissue-specific expression patterns of connexins and their transcriptional activators and repressors.

1.6.3 Functional roles of GJIC

Research of gap junctional communication is significant in many fields of biology, since GJIC can influence cellular proliferation, differentiation, and invasion. Since these processes are integral for many aspects of developmental biology, physiology, cancer biology, and other pathophysiologies, it has become increasing obvious that GJIC plays an obligate role in cellular and tissue function. Still, the specific physiological functions that connexin channels serve *in vivo* have been demonstrated in only a few select tissues. For example, it is now accepted that GJIC plays a regulatory role in the electrical and mechanical coupling of different types of muscle cells, including the heart, but for the most part the endogenous signals that pass through gap junctions have not been determined. The following section will discuss research of GJIC in terms of cancer, development, and adult tissue function, as well as human diseases that have been associated with abnormal GJIC.

GJIC and Development

Gap junctions have been implicated in many development processes in various species because of their embryonic tissue-specific patterns of expression. There has been tremendous insight into the role for gap junctions in development by the generation of mice with null mutations in connexin genes. These knock-out phenotypes are summarized in Table 1.2. Of interest for the present study are the phenotypes of the Cx40, Cx43 and Cx45 null mice. As will be presented in Chapter 2, Cx40 and Cx45 are expressed in anchoring trophoblast cell columns in the early human placenta. However, it is important to note that these two connexins are not expressed in equivalent cell-types in the mouse placenta – in fact Cx40 is only expressed in placental vascular endothelial cells and Cx45 is expressed only in extra-embryonic cells of mesodermal origin, and not of epithelial origin (from which trophoblasts are derived). Cx40-deficient mice display reduced cardiac conduction velocity and a predisposition to arrhythmias, since this connexin is known to be expressed in cells of the atrial ventricular (AV) node and cells of the conductive system in the myocardium (166). In Cx45-deficient mice there is defective vascular development, and these mice die between embryonic day 9.5 and 10.5 pc (167). More specifically, although the differentiation and positioning of endothelial cells appeared to be normal, subsequent development of blood vessels reveals impaired formation of vascular trees in the yolk sac, and arrest of arterial growth, including a failure to develop a smooth muscle layer

Table 1.2. Phenotypes of Mice Deficient in Connexin Genes (adapted from Novartis Foundation Symposium on Gap Junctions, 1999).

Cx26	embryonic lethal at day 11 pc *in utero* because of decreased glucose transfer between syncytiotrophoblast layers I and II in the placenta
Cx31	viable and fertile, greater post-implantation blastocyst lethality than expected by Mendelian inheritance
Cx32	viable and fertile, smaller than wild-type littermates by 17%, impaired glucose mobilization from hepatic glycogen stores, decrease in hepatic Cx26 expression, peripheral neuron abnormalities, increased incidence of spontaneous and chemically induced liver tumours
Cx37	viable but females are infertile, fail to ovulate and develop several inappropriate corpora lutea. Oocyte development is arrested before meiotic competence, no effects on endothelial cells of Cx37-deficient mice have been reported to date
Cx40	viable and fertile, reduced atrio-ventricular conduction, increased incidence of arrhythmias
Cx43	lethality shortly after birth, obstruction of the right ventricular outflow tract of the heart, improper neural crest cell migration
Cx45	embryonic lethal at day 9.5 pc because of outflow tract dilation, right ventricular hypertrophy, yolk sac vasculature abnormalities, enlarged placental labyrinth embryonic vessels, general vascular abnormalities
Cx46	viable and fertile, develop nuclear lens cataracts at 2-3 weeks of age coincident with accumulation of γ-crystallin
Cx50	viable and fertile, small eyes and lenses compared to wild-type littermates (56%), develop nuclear opacity after one week of age

surrounding the major arteries of the embryo proper. The phenotypes of both the Cx40 and Cx45 mice do not reveal any information about these connexins in mouse placental development, but as will be discussed later in this section, the expression of connexins in the mouse placenta is very different from expression in the human placenta.

An interesting connexin-deficient mouse in terms of this project is the $Cx43^{-/-}$ mouse. Homozygous Cx43 knockout mice die at birth from pulmonary outflow tract obstruction and conotruncal heart malformations (168). Interestingly, Ewart *et al.* have also observed conotruncal heart defects in transgenic mice overexpressing Cx43 (169). Cecelia Lo's group proposes that these findings suggest that the level of Cx43-mediated gap junctional communication may play an important role in conotruncal heart development. Cardiac neural crest cells emanate from the postotic hindbrain neural fold in the developing mouse and then migrate in groups of cells organized in streams or sheets to the outflow tract area of the heart (170). Cardiac crest cells endogenously express Cx43, and in Cx43-deficient mice cardiac crest cells do not properly migrate to the outflow tract (171). Based on this and many other observations, Lo has suggested that gap junctions may mediate cell-cell signaling among a stream of migrating crest cells and thus allow the rapid integration of signals from multiple cell signaling pathways. In this manner, the migratory behaviour and/or function of crest cells can be uniquely and efficiently specified by a mixture of environment cues. Their current model is that second messengers arising from cell signaling cascades triggered by receptor-ligand interactions at the cell surface may move from cell to cell through a crest migratory stream via gap junctional channels, and that this may serve to coordinate the migration, differentiation, and/or proliferation of cardiac crest cells (172). Since trophoblast cells in anchoring columns also form a migratory group of cells, it was tempting for us to speculate that gap junctions may also be required in this cell population to coordinate and integrate signaling events.

Another important role for gap junctions during development is in left-right patterning. Using both *Xenopus* and chick embryo models, Levin and Mercola have determined that gap junctional communication is imperative for normal patterning (173, 174). During left-right patterning, different cascades of asymmetric genes that distinguish the left and right sides of the embryo are maintained by a midline barrier. Prior to the formation of this distinct left-right barrier, gap junctional communication is required to keep left-side genes to the left and right-side genes to the right. When Levin *et al.* treated chick embryos with lindane, a gap junctional

inhibitor, there was unbiased expression of the normally asymmetric left-right expression of *Shh* and *Nodal*, causing the normally left-sided program to be recapitulated symmetrically on the right side of the embryo. Cx43 mRNA is present in the chick blastoderm prior to any known asymmetric gene expression. Specifically inhibiting Cx43 by either antisense oligonucleotides or a blocking antibody in cultured chick embryos also results in bilateral expression of *Shh* and *Nodal* transcripts. Levin *et al.* propose a model in which unknown left-right determinants are transferred unidirectionally around the blastoderm through gap junctions to produce a gradient, with low molecular mass determinants preferentially accumulating on one side of the midline. Whether this requirement for GJIC in left-right patterning is maintained in mice has not yet been determined, since all knockout mice produced so far do not display any left-right asymmetry alterations. It is possible that the lack of this phenotype in knockout mice is due to compensation or redundancy of connexins in the mouse.

Changes in GJIC have also recently been shown to directly influence differentiation processes of various cell-types. For example, Li *et al.* have determined that inhibiting GJIC alters the expression of differentiation markers in osteoblastic cells (175). When rat osteoblastic cells are treated with anti-sense cDNA for Cx43, there is a decrease in alkaline phosphatase activity and osteocalcin levels, two phenotypic characteristics of mature osteoblastic cells. Even more intriguing are studies performed by Christian Naus's group, in which myoblasts treated with GJIC-inhibitors also undergo changes in their normal *in vitro* differentiation process (176). Normally cultured myoblasts can be induced to differentiate into myotubes by serum withdrawal. This process has been shown to require the bHLH transcription factors myogenin and MRF4, which increase upon differentiation. When they treated myoblasts with the known GJIC-inhibitors octanol and β-glycyrrhetinic acid, the activation of myogenin and MRF4 was also inhibited, and morphologic differentiation was also abrogated. These data suggest that the inhibition of GJIC can alter the activities of essential transcription factors in differentiation processes. The mechanisms that may be involved in this have not yet been determined. A similar phenomenon may be a component of trophoblast differentiation, since bHLH transcription factors are also essential to this process.

GJIC and Physiology

Since connexins are expressed in all tissues, it is beyond the scope of this thesis to discuss the proposed functions of gap junctions in all tissues. Therefore, this section will focus on connexin expression and function in three systems: the nervous system, the heart, and the myometrium.

Gap junctional coupling is widespread throughout the developing mammalian nervous system, among both neurons and glial cells (reviewed in (177)). Roles for GJIC in mediating neuronal pattern formation, cell migration, neuronal differentiation, and circuit formation have been proposed (178, 179). In the adult nervous system, gap junctional coupling seems to be restricted primarily to specific groups of neurons. In the rodent cerebral cortex, neurons are transiently coupled during development and for the first 2 weeks of life, and although neurons in adults express connexins, they are not functionally coupled. In the rat brain, these first 2 weeks of life are when the morphological and functional differentiation of neurons are highest. One possibility for the need of GJIC during this period is that it may serve to guide chemical synapse formation with groups of neurons rather than with individual cells. Therefore, similar to neural crest cells, neurons also appear to require coupling in order to act in a developmentally coordinated manner.

In the cardiovascular system, there are 3 main connexins: Cx43, Cx40, and Cx45. The predominant connexin of cardiac muscle is Cx43, found in abundance in the working ventricular and atrial myocardium of all mammalian species (180, 181). Cx40 is typically expressed in atrial muscle cells and in specialized cells of the ventricular conduction system (conducting bundle branches and Purkinje fibers) (182). *In vitro*, gap junctions composed of Cx40 have been shown to have high conductance values (typically 160-200 pS, compared to 60 pS for Cx43 channels), which is thought to contribute to the fast conduction properties of the ventricular conduction system (183). Finally, Cx45 co-localizes with Cx40 in the ventricular conduction system (168), which provides an explanation for the observation of first degree atrioventricular block (in which impulses continue to reach the ventricle but are delayed), rather than complete AV-block in Cx40-deficient mice. Thus, the overall role of connexins in the heart is for the coordination of electrical impulses originating from the AV node, through the conduction system of the ventricles and around each ventricle with every heartbeat.

In the rodent myometrium, there is a regulated pattern of connexin expression during pregnancy. The connexins that are expressed in the rat myometrium during pregnancy include Cx43, 26, 37 and 40 (184). Cx43 expression is low throughout pregnancy but increases immediately before the onset of labour (day 23). Cx26, on the other hand, increases on day 17 of rat pregnancy, reaches maximal levels between days 19 to 21, and falls to low levels prior to the onset of labour. Furthermore, if pregnant rats are treated with progesterone, which blocks both Cx43 induction and the onset of labour, Cx26 mRNA levels are maintained, indicating differential hormonal regulation of Cx43 and Cx26 transcription. Gap junctions comprised of Cx43 in the labouring rat myometrium are thought to synchronize the spread of contractions required for the expulsion of the fetuses. The role for Cx26 just prior to the onset of labour is speculative at the moment. It has been suggested that once small gap junctional plaques are formed, new channels may form more readily by recruitment to these plaques, and that this foundation favours the formation of large plaques of gap junctions (185). Thus, it is possible that myometrial gap junctions comprised of Cx26 provide an underlying foundation for the subsequent production of the large Cx43-containing plaques which are characteristic of labour.

GJIC and Cancer

In 1966, W.R. Loewenstein postulated that cancer was the result of dysfunctional GJIC (186). Although there has been an exponential increase in knowledge surrounding the process of carcinogenesis since then, this early hypothesis has been supported by numerous reports. It is thought that a down-regulation of GJIC could lead to dysregulated cellular growth by isolating cells from their neighbours. Gap junction number, connexin expression, and cell-cell coupling have been studied in many neoplastic cells by multiple means, and the vast majority have fewer and smaller gap junction plaques, express less connexins, and have reduced GJIC compared to their non-neoplastic counterparts (187). However, there are exceptions – some tumour cells have normal or even greater than normal gap junction expression. It remains to be determined whether these are functional gap junction channels *in vivo*, and even if they are it is possible that they can form homotypic gap junction channels amongst cancerous cells, but not heterotypic channels with surrounding nontransformed cells. This latter possibility has been substantiated by several groups who have identified several neoplastic lines that have extensive gap junction formation, connexin expression, and homologous GJIC, but little heterologous GJIC with their

nontransformed counterparts (187). This inability of tumour cells to communicate with normal cells is not due to the expression of different connexins, but possibly to differences in cell surfaces (eg. glycosylation or cell-cell adhesion molecules) that prevent adequate cell-cell contact needed for gap junction formation.

Many known chemical carcinogens have been identified that promote neoplastic transformation through mechanisms that do not involve direct damage of DNA. These agents are able to selectively induce the proliferation and/or inhibit apoptosis of preneoplastic cells. This leads to the clonal expansion of the preneoplastic cell population and increases the risk of additional genetic changes that could result in full neoplastic transformation. GJIC may play a role in this proliferative response. Most of the tumour promoters that have been examined (over 100) inhibit GJIC in cultured cells and cells within target tissues. In fact, the ability of tumour promoters to inhibit GJIC is one of their most common properties. Connexins can therefore be thought of as tumour-suppressors. In a study by Huang *et al.*, human brain glioblastoma tumour cells were investigated for expression of Cx43 and GJIC (188). Western blot and RT-PCR analysis indicated that the expression levels of Cx43 are profoundly decreased in several of the cell lines they examined. Transfection of human Cx43 into these human glioblastoma cell lines dramatically reduced cell proliferation, but these effects were not associated with the establishment of functional GJIC. These data suggest that there is a role for connexins as tumour-suppressors independent of cell-cell coupling. In HeLa cells, which do not express detectable levels of connexins, transfection of Cx26, but not Cx40 or Cx43, results in the suppression of the tumourigenic phenotype of the HeLa cells both *in vitro* and *in vivo* (in mice). In an interesting study by Omori *et al.*, HeLa cells were transfected with Cx26 and truncated forms of Cx43 and Cx32 lacking their C-terminal cytoplasmic domains. Cx26 has a very short C-terminal cytoplasmic domain but Cx43 and Cx32 have relatively long ones (189). Surprisingly, they found that the transfection of truncated Cx43 and Cx32 (which would mimic Cx26 structure) also resulted in a reduced invasive capacity of the HeLa cells. Therefore, the presence of the large cytoplasmic domains in wild-type Cx43 and Cx32 prevented their actions as growth control tumour-suppressors in HeLa cells.

The amount of functional GJIC is also inversely related to invasive capability of some tumour cell-types: the more coupled the cells, the less invasive. McDonough *et al.* determined the existence of such a relationship in glioma cells isolated from different regions of a glioma-

bearing dog brain (190). They found variable levels of GJIC amongst cell lines propagated from these samples, and that lines with a reduced capacity to connect to each other have an accelerated invasion rate onto glioma-derived matrix. Based on this role for a decrease of GJIC in tumour invasion, it could also hold true that placental trophoblast invasion depends on a decrease in GJIC. This hypothesis was addressed in this thesis project.

Other Human Diseases Associated with Abnormal GJIC

The association of human genetic diseases with specific connexin mutations has also provided information on the role of gap junctions in diverse physiological processes. The human disorders identified so far are summarized in the following table (Table 1.3). The first disease-associated connexin was Cx32, in the X-linked form of Charcot-Marie-Tooth disease, a progressive neuropathy resulting from myelin disruption and axonal degeneration of peripheral nerves (191). Cx32 mutations are suggested to impair the diffusion of metabolites through gap junctions that are found between the Schwann cell body and its distal processes. Considering the preponderance of this connexin in the liver, it is surprising that Cx32 mutations do not cause major liver dysfunction, suggesting that other connexins compensate in the liver.

The inner ear and the skin have abundant gap junctions, and many of the connexin mutations in humans are associated with deafness and skin disease. During auditory transduction in the inner ear, gap junctions are proposed to maintain membrane potentials by regulating ionic flow (specifically of potassium ions) between the sensory epithelial of the inner ear (192). Mutations in Cx26, Cx30 and Cx31, all of which are expressed in the inner ear, are associated with non-syndromic (single phenotypic disease) sensorineural hearing loss (192). Gap junctions are also thought to coordinate keratinocyte differentiation (193), and mutations in Cx26 and Cx31 have been shown to cause dominant disorders of epidermal keratinization and the skin disease erythrokeratoderma variabilis (EKV), respectively. The mutations in Cx31 are different between deafness and EKV, and another distinct Cx31 mutation segregating with both deafness and neuropathy has also recently been identified, indicating that three distinct mutations in one gene can cause three diseases (194). There has not been any reported placental dysfunction associated with mutations in connexins.

Table 1.3. Human Diseases with Mutations in Connexin Genes (adapted from Kelsell *et al.*, 2001).

[illegible]	X-linked Charcot-Marie-Tooth Disease: neuropathy often associated with hearing loss
[illegible]	Dominant and recessive non-syndromic moderate to profound sensorineural hearing loss Dominant epidermal disease (palmoplantar keratoderma and Vohwinkel's syndrome)
[illegible]	Recessive non-syndromic moderate to profound sensorineural hearing loss Dominant non-syndromic high frequency hearing loss Dominant skin disease (Erythrokeratoderma variabilis) Dominant sensorineural hearing loss and neuropathy
[illegible]	Dominant skin disease (Erythrokeratoderma variabilis)
[illegible]	Dominant hearing loss Clouston's hidrotic ectodermal dysplasia: skin disease (palmoplantar keratoderma), hair loss, nail defects and often mental deficiency
[illegible]	Association with visceroatrial heterotaxy
[illegible]	Dominant zonular pulverant cataract
[illegible]	Dominant zonular pulverant cataract

1.6.4 GJIC conductance gating

The conductance of single gap junction channels, and consequently of plaques as a whole, can be altered by various mechanisms, including changes in pH, calcium concentrations, and by phosphorylation. These all affect the gating of the channels, which is defined as the reversible transition between open and closed conformations of pre-formed channels. Connexin channels are also voltage-sensitive; the kinetics and steady-state properties of voltage-dependence differ enough among members of the connexin family to indicate that a wide range of voltage gating behaviours exist (195). Since there are no known substances that can increase the open-state probability of intercellular channels, gating of gap junction channels involves the closure of channels.

All connexins exhibit a variable degree of sensitivity to experimental treatments inducing intracellular acidification (196). Experiments with truncated constructs and site-directed mutagenesis have revealed that the length of the primary sequence of the middle cytoplasmic loop and carboxy-tail play a critical role in pH gating. For example, Cx43 mutants in which the histidine at position 95 is replaced with acidic or uncharged residues are less susceptible to acidification, whereas the opposite is true when the same histidine is substituted with a basic residue (196). Based on this and many other studies, it appears that the middle and C-terminal cytoplasmic loops may participate in a ball-and-chain type gating interaction, which closes in low pH conditions.

Calcium is also known to modulate gating of Cx43 gap junction channels, independent of the cell-type which expresses it (197). A depression of junctional conductance becomes significant at around 400-500 nM calcium, which is within physiologic ranges (100nM to μM). A direct effect of calcium seems unlikely, as connexins do not possess consensus sequences for putative calcium-binding sites. Based on its ability to bind to Cx32 and the presence of possible binding sites on connexins, the ubiquitous calcium-receptor protein, calmodulin, has been considered a logical mediator of the calcium effect (198).

Another likely effect of calcium that could modulate gap junctional gating is through the activation of specific kinases and subsequent connexin phosphorylation. Protein phosphorylation on serine, threonine or tyrosine residues is a well-characterized mechanism by which extracellular signals can significantly alter the function of ion channels. Except for Cx26,

that has neither consensus sequences for kinases, nor is phosphorylated by protein kinase A (PKA), protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (199), all other connexins which have been directly examined are phosphoproteins, including Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, Cx45.6, Cx46, Cx50 and chCx56 (200). Cx43 is known to be differentially phosphorylated in a tissue-specific manner, and this constitutive phosphorylation has been correlated to the communication competence of some cell lines (201, 202). The expression of individual connexins in transfected cell lines has now demonstrated that connexin channels are in a more 'open' configuration with dephosphorylating treatments (203). Many kinases are able to phosphorylate Cx43, including $pp60^{src}$, which phosphorylates on tyrosine (204); and PKC (205), MAP kinase (206), the cyclin B-dependent kinase $p34^{cdc2}$ (207), and cAMP-dependent protein kinase (PKA), which phophorylate on serine or threonine residues. Cooper *et al.* estimate that Cx43 and Cx45 are routinely phosphorylated in unstimulated cells on at least five and three serine residues, respectively (208). In the Cx45 carboxy-tail, there are 9 serine residues that can be modified by phosphorylation, but the role of each of these phosphorylations *in vivo* remains unknown (209).

Because of the existence of multiple phosphorylation sites on many connexins and the many kinases that can phosphorylate these sites, the level of connexin phosphorylation in response to multiple ligands may be integrated to modulate GJIC. The relevance of this in placentation will be discussed in Chapter 3, since many of the growth factors that are implicated in trophoblast differentiation have also been shown to result in the phosphorylation of various connexins, which could affect GJIC.

1.7 Gap Junctions in Placental Development

The expression of connexins in the rat placenta and endometrium during implantation and early development has been elucidated, and spatial and temporal changes in connexin expression have been revealed (27). As the blastocyst implants in the rat endometrium (at day 7 pc), the ectoplacental cone expresses Cx31. Between day 10 and 14 pc, Cx31 is spatially restricted to the invasive and proliferative spongiotrophoblast, but expression of Cx31 is lost at day 14 and the fully differentiated spongiotrophoblast expresses Cx43 instead. In addition, Cx26 is induced in the differentiated labyrinthine trophoblasts during the second half of pregnancy. Cx26 gap junctions have been suggested to serve as channels for nutrients between maternal and fetal

blood. Shin *et al.* have immunolocalized Cx26 between STB layers I and II, whereas the glucose transporter protein GLUT1 is located in the plasma membrane on the maternal side of STB I and on the fetal side of STB II (210). The hypothesis that glucose transfers from mother to fetus requires transport through Cx26 channels was addressed by the generation of mice with null mutations in this gene. The Cx26 null mouse dies *in utero* at day 11 pc, and does not express Cx26 in the labyrinthine region of the placenta. When Gabriel *et al.* measured embryonic uptake of the nonmetabolized glucose analogue 3-O-[^{14}C]methyl-glucose injected into the maternal tail vein, Cx26$^{-/-}$ mice accumulated only 40% of the radioactivity measured in wild-type and heterozygous littermates (211).

Human placental development is anatomically different from that of rodents, and the role of gap junctional intercellular communication and the complete expression patterns of human connexins in the developing placenta have not been fully determined. Gap junctions have been implicated in the differentiation of stem CTBs through both differentiation pathways - towards STB and towards invasive EVT cells. Khoo *et al.* have transformed the normal trophoblast cell line HTR8 by *SV40 Tag* transformation and described changes in Cx43 expression and GJIC (212). The HRT8 parental cell line was analyzed for expression of Cx26, Cx32, Cx40 and Cx43 by immunofluorescence and for transcripts of Cx32, Cx26, Cx33 and Cx43 by Northern blot, and only Cx43 was detectable. Upon immortalization of HTR8 cells by *SV40 Tag* transformation to produce the immortal RSVT2/C line, no detectable Cx43 was observed. Furthermore, GJIC was diminished in the transformed line, as determined by a dye transfer assay. These studies agree with the hypothesis of connexin-mediated growth control and its abrogation during tumour progression, since RSVT2/C cells display both a hyperinvasive phenotype and decreased GJIC. This suggests that *in vivo*, trophoblast differentiation towards the invasive phenotype may also involve a down-regulation of GJIC, which was addressed using an *in vitro* model in this thesis project.

Elke Winterhager's lab has been interested in connexins during rodent placental development for many years, and more recently they have also investigated connexins in the early human placenta. In 1999, her group published a study that screened for the expression of 6 connexin proteins in first trimester human placentas by immunofluorescence (Cx26, Cx31, Cx32, Cx37, Cx40 and Cx43) (213). The studies will be discussed further in Chapter 2, since similar experiments were performed for this thesis project. A role for connexins in human trophoblast

invasion can also be inferred from the presence of Cx40 in the human choriocarcinoma cell lines Jeg3, BeWo and Jar (although Jeg3 cells express extremely low levels) (214).

Other groups have been interested in the role of gap junctions during syncytialization, since it has been observed since 1975 that cells undergoing fusion form gap junctions. In isolated CTBs, Cx26, Cx32 and Cx40 cannot be detected, but Cx43 is expressed at both the mRNA and protein level (215). When these cells are stimulated to fuse *in vitro*, Cx43 staining is only observed in the cytoplasm of the cells, and not on the membrane. Cronier *et al.* have been able to detect Cx43 between villous CTB and STB in first trimester placenta *in situ* (216). When they treated isolated trophoblast cells with heptanol, a GJIC-inhibitor, there was a dramatic decrease in the number of coupled cells and STB formation, as well as hCG and hCS secretion, indicating that GJIC is required for syncytialization.

1.8 Rationale and Hypothesis

Rationale

Based on the literature review presented in this chapter, we speculate that gap junctions play an important role in trophoblast differentiation along the invasive pathway. In anchoring column EVT cells, gap junctions may be required to coordinate migration of the column into the decidua, a role analogous to that of neural crest cell migration in the developing mouse. EVT cells at the distal leading edge of the column could transduce signals from various cytokines/growth factors that interact with receptors on these cells along the length of the column, thus keeping all of the cells together and organized. The presence of gap junctions in proliferative EVT cells may also be involved in maintaining proliferation, since proliferating cells in culture express connexins (217), and based on evidence from proliferative tumour cells that have abundant homotypic GJIC. We therefore sought to determine if connexins are expressed in EVT cell columns in the first trimester human placenta, and specifically which connexin sub-types are expressed in this population.

The invasive capacity of tumour cells, including those of trophoblast origin (choriocarcinoma), is inversely related to cell-cell coupling. Invasiveness is high when GJIC is low. Since EVT cells at the distal tips of anchoring columns must differentiate into an invasive phenotype, a down-regulation of GJIC at this point may facilitate EVT cells to 'let go' of the

column and subsequently invade the decidua as individual cells. We chose to examine the role of GJIC in EVT cell differentiation using the placental villous explant culture system, which has been characterized by other groups (31, 76, 136, 218). To determine if the down-regulation of GJIC may be involved in the EVT differentiation process, we used three GJIC-inhibitors with different chemical structures and mechanisms of action. To assess whether GJIC-blockade resulted in a change in EVT cell phenotype to a non-proliferative/invasive phenotype, we characterized the phenotype obtained upon treatment with the GJIC-inhibitors.

Hypothesis

We hypothesize that connexins are expressed in anchoring EVT cell columns *in vivo* and in placental villous explant outgrowths in culture. Since we propose that GJIC and/or connexin expression is down-regulated at the distal edge of columns, we hypothesize the inhibition of GJIC in the explant culture system may alter the phenotype of the EVT cells from the proliferative to an early-invasive phenotype.

The specific **aims** of this thesis project were:

1. To determine by RT-PCR which connexin transcripts are expressed in the first trimester human placenta. (Chapter 2)
2. To evaluate changes of the connexin transcripts present in the placenta throughout the first gestation (5-14 weeks) by semi-quantitative RT-PCR. (Chapter 2)
3. To localize these connexins in the first trimester human placenta by immunofluorescence, immunohistochemistry or *in situ* hybridization. (Chapter 2)
4. To localize these connexins in first trimester villous explant cultures grown in 3% O_2 by immunofluorescence, immunohistochemistry or *in situ* hybridization. (Chapter 3).
5. To determine the effects of inhibiting GJIC in first trimester placental villous explant cultures by treatment with three known GJIC-inhibitors, carbenoxolone, heptanol, and oleamide. (Chapter 3) Explants were assessed for outgrowth EVT proliferation by Ki67 immunostaining, for MMP activity by zymography, for integrin expression by immunostaining, as well as morphologically and histologically.

6. To determine the effect of specifically 'knocking-down' Cx40 expression in villous explant culture using anti-sense oligonucleotides. In these experiments, changes in villous explant outgrowth morphology were determined. (Chapter 3)

CHAPTER 2

CHARACTERIZATION OF CONNEXIN EXPRESSION IN THE EARLY HUMAN PLACENTA

2.1 Introduction

Gap junctional intercellular communication (GJIC) is known to modify cell proliferation, migration, and invasion. In human placental development, these processes are essential for placental formation and function, and aberrant trophoblast proliferation and invasion can lead to clinically relevant diseases such as choriocarcinoma (resulting from trophoblast hyperproliferation and over-invasion) or preeclampsia (resulting from shallow trophoblast invasion). The early human placenta is composed of hundreds of villi, some of which are in contact with the maternal decidua via anchoring cell columns composed of proliferating EVT cells. EVT cell columns migrate into the decidua, and at the distal edge EVT cells stop proliferating and differentiate into an invasive phenotype. Invasive EVT cells individually invade into the decidua until they reach maternal arteries, where they replace endothelial cells surrounding these vessels. This remodeling by EVT cells results in vasodilation, and hence an increase in blood flow into the intervillous space bathing placental floating villi. Nutrients and waste products can then be transferred from the maternal blood pool directly into the placenta and to the developing fetus.

Gap junctions are required in neural crest cell migration during early rodent development. Neural crest cells arise as an outgrowth from the neuroectoderm of the neural tube and migrate in streams or sheets to selective destinations, including the outflow tract of the heart (219). This migration process is analogous to EVT cell column migration in the developing placenta, which led us to investigate the expression of gap junction proteins (connexins) in the early human placenta. Placentas were first screened by RT-PCR to identify the connexin transcripts that are expressed during this critical period of placental development. The connexins for which primers were designed for this purpose were human Cx26, Cx31, Cx32, Cx37, Cx40, Cx43, Cx45, and rat Cx46, since the human sequence was not available. The only known human connexins missing from this screen at the initiation of this project were Cx31.1, which is restricted to skin keratinocytes (220), and Cx50, which is restricted to the lens of the eye (221). For those connexins identified in the placenta, semi-quantitative RT-PCR was performed on samples spanning the first trimester, from 5 to 14 weeks gestation. Originally, this was done with the rationale that a decrease in the expression of one or more connexins at 9-10 weeks gestation may be indicative of a role for those connexins in trophoblast invasion, since some investigators

believe that trophoblast invasion is 'maximal' at this period, when oxygen tension increases in the placenta (218). If connexins are required to maintain proliferative EVT cell columns, then at the time of 'maximal' invasion we would expect connexin expression to be down-regulated in order to allow for invasion. However, other models propose that trophoblast invasion is a continuous process, occurring throughout the first trimester and into the second trimester as well (135). The only way to ascertain that connexins may be pivotal in EVT function was to localize expression patterns in the early human placenta. To this end, immunofluorescence, immunohistochemistry, and *in situ* hybridization experiments were performed on first trimester human placental sections. It was of great interest for us to determine whether any connexins were expressed in EVT cell columns, where GJIC may mediate organized migration of the column into the decidua.

2.2 Materials and Methods

Human first trimester placenta samples

First trimester placentae were obtained at the time of elective terminations of pregnancy from patients admitted to Mount Sinai Hospital, Toronto, Canada. Informed consent was obtained from each patient and collections were approved by the University of Toronto's Review Committee on the Use of Human Subjects. Placental tissue from 5-14 weeks of gestation was dated according to the criteria of the Carnegie classification evaluating characteristics of embryonic/fetal parts. Placental tissue was washed in ice-cold PBS, and amnion and umbilical cord were dissected away. Each placenta was then dissected into two, one half was flash frozen and stored at -70°C for future use, and the other was immediately fixed in 4% paraformaldehyde for immunohistochemistry and *in situ* hybridization. Fetal parts were also flash frozen and stored at -70°C for use as a control tissue, since they express many connexins.

RT-PCR and Semi-quantitative RT-PCR

Frozen first trimester placental tissues and control tissues were pulverized in liquid nitrogen and homogenized in 1 mL Trizol RNA extraction reagent per 100 mg of tissue on ice. Total RNA was extracted according to the manufacturer's specifications. RNA quality was

monitored by UV spectrophotometry and by electrophoresis of each RNA sample on a 1.5% agarose gel. Complementary DNA (cDNA) was generated in a 150 μL reaction containing 7.5 μg total RNA with 1500U M-MLV reverse-transcriptase (GibcoBRL) according to the manufacturer's protocol.

Primers targeted specifically for human Cx26, Cx31, Cx32, Cx37, Cx40, Cx43, Cx45 and rat Cx46 were used for amplification of cDNA from human first trimester placentas. Primer sequences, optimum annealing temperature and expected length of amplified product are summarized in Table 2.1.

Standard PCR was performed in 35 μL reactions containing 5.25 μL of RT sample using 1.5U *Taq* DNA Polymerase (GibcoBRL). The amplification profile involved preincubation at 94°C for 5 min, denaturation at 94°C for 30 sec, primer annealing at the optimal annealing temperature of each primer pair for 30 sec, and extension at 72°C for 45 sec over ≥ 30 cycles. PCR products were electrophoresed on 1.5% agarose gels in Tris-acetate/EDTA buffer in order to visualize amplification products. As a negative control, template RT was omitted from the PCR reaction, which resulted in the absence of any amplification product. Also, RNA was used as template to ensure that there was no DNA contamination.

For semi-quantitative RT-PCR, optimal conditions were determined for each pair of primers by adjusting the following PCR reaction parameters: annealing temperature (between 50-65°C), $MgCl_2$ concentration (0.75-2 mM), and dNTP concentration (0.15-0.35 mM), using pooled first trimester RT as template. Following this, PCR amplification was followed over multiple cycle numbers in order to plot the linear range of amplification for each primer pair (between 20 and 35 cycles at 3 cycle intervals). Once conditions were optimized, for each RT sample PCR was performed in triplicate at 3 cycles within the linear range. Thus, PCR was conducted at 26, 28, and 30 cycles for Cx32; 26, 28 and 30 cycles for Cx37; 22, 24, and 26 cycles for Cx40; 23, 25 and 27 cycles for Cx43; and 26, 28 and 30 cycles for Cx45. As an internal standard, β-actin was also amplified in each RT sample over 3 cycles within its linear range (cycles 17, 19 and 21). For each connexin primer pair, at least 3 placentae in each age grouping were analyzed. All samples were electrophoresed on a single gel, stained with ethidium bromide, photographed using Polaroid 665 Positive/Negative film, and quantified by densitometry using ImageQuant software. The calculation of relative transcript abundance for each connexin in each placental RT sample was carried out as follows, using Cx43 as an

Table 2.1. Connexin primers, optimal annealing temperature, and expected length of amplification product.

[redacted]	upper 5'-GAA GTT CAT CAA GGG GGA GAT-3' lower 5'-CTT TGT GTT GGG AAA TGC TG-3'	63	474
[redacted]	upper 5'-GGT TAG GCT GGG GCT GAT G-3' lower 5'-GTT GGT TAG TGC GGG AGA TGA-3'	61	508
[redacted]	upper 5'-ACC AAT TCT TCC CCA TCT CC-3' lower 5'-CTG GTA TGT GGC ATG AGC A-3'	60	386
[redacted]	upper 5'-GAC CAT GGA GCC CGT GTT TGT-3' lower 5'-GGG ACG ACT TGG GGG TTT TTG-3'	63	433
[redacted]	upper 5'-CCG GCC CAC AGA GAA GAA TGT-3' lower 5'-TCT GAC CTT GCC TTG CTG CTG-3'	61	465
[redacted]	upper 5'-AAA GAG ATC CCT GCC CAC ATC-3' lower 5'-GCC CAG TTT TGC TCA CTT GCT-3'	61	370
[redacted]	upper 5'-CAA GTC CAC CCG TTT TAT GTG-3' lower 5'-AGT TCT TCC CAT CCC CTG AT-3'	60	574
[redacted]	upper 5'-GGA AAG GCC ACA GGG TTT CCT GG-3' lower 5'-GGG TCC AGG AGG ACC AAC GG-3'	62	332
[redacted]	upper 5'-GGA AAT CGT GCG TGA CA-3' lower 5'-GTA CAG GTC TTT GCG GAT GT-3'	61	265
[redacted]	upper 5'-GGC CAG GTC ATC ACC ATT G-3' lower 5'-TGT GTG GAC TTG GGA GAG GAC-3'	61	748

example. For one placenta sample, the densitometric values obtained at cycles 23, 25 and 27 were plotted, and by linear regression analysis a value was obtained for 25 cycles (the middle cycle number). This value of raw optical density (ROD) was normalized to the ROD for β-actin at 19 cycles obtained in an identical manner, yielding the relative expression level of Cx43 mRNA in that sample.

Plasmid construction

Plasmids containing connexin PCR products were constructed using a TA Cloning Kit (Invitrogen). PCR amplification products were extracted from agarose gel slices using a column extraction kit (Qiagen). Purified PCR products were then ligated into vector pCR2.1 and transformed into competent *E. coli*. Positive clones were identified by appropriate restriction digest analysis. Connexin PCR fragments were then sub-cloned into pBluescript, which contains both T7 and T3 RNA polymerase promoters, by digestion of both plasmids with HindIII and XbaI. Following ligation (overnight, 14°C), competent *E. coli* were transformed and positive clones were identified by restriction digest analysis and confirmed by sequencing. Plasmids containing PCR fragments of Cx32, Cx37, Cx40 and Cx45 were constructed in pBluescript in this manner for use as templates for sense and anti-sense probe labeling and subsequent *in situ* hybridization experiments.

Immunohistochemistry and Immunofluorescence

For immunolocalization experiments, either immunohistochemistry or immunofluorescence techniques were used. Fresh placental and control tissues were fixed in cold 4% paraformaldehyde for 4 hours at room temperature or overnight at 4°C. The tissues were then washed in PBS, and dehydrated for paraffin-embedding through a gradient series of ethanol in PBS up to 100% ethanol. Samples were cleared in xylene for 1 hour, and infiltrated with paraffin for 4 hours or overnight. Paraffin embedded samples were sectioned at 6 μm using a microtome. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated through a gradient series of ethanol in PBS. Endogenous peroxidase activity was blocked by incubation of the sections in 3% H_2O_2 in methanol for 20 min. Following PBS washes, antigens were unmasked using an optimal antigen retrieval treatment determined for each antibody (0.125% trypsin, 5-10 μg/mL proteinase K, 10 mM sodium citrate, or 0.02-0.2% Triton-X100).

Sections were washed 3 times in PBS for 5 min, and nonspecific binding sites were blocked in a solution containing 5% normal goat serum and 1% BSA in PBS at room temperature for 30 min. Primary antibody dilutions are summarized in Table 2.2.

Table 2.2. Primary antibody dilutions.

HLA-G	mono	1:20	gift from Jay Cross, SLRI
Cx32	poly	1:200	gift from Elke Winterhager, University of Essen
Cx40	poly	1:100	gift from Elke Winterhager
Cx43	poly	1:150	produced in the Lye lab
Cx45	poly	1:50	gift from Elke Winterhager

Incubations with primary antibodies were carried out at room temperature for 2 hours or overnight at 4°C. Following three 5 min washes with PBS, sections were incubated with biotinylated anti-mouse (1:300) or anti-rabbit (1:750) IgG (Vector Laboratories) diluted in blocking solution for 1 hour at room temperature. After washing 3 times with PBS, slides were incubated with an avidin-biotin complex (Vector Laboratories) for 1.5 hours. Slides were washed again in PBS and developed in 0.075% (wt/vol) 3,3-diaminobenzidine in PBS containing 0.002% (vol/vol) H_2O_2 with or without nickel enhancement (Vector Laboratories), to yield a black product (with nickel enhancement) or a brown product (without nickel enhancement). After light counterstaining with Alcian Blue/Methyl Green (Sigma), slides were dehydrated in an ascending ethanol series, cleared in xylene and mounted with Permount (Fisher). In control experiments, primary antibodies were replaced with blocking solution.

For immunofluorescence, sections were treated following the same steps as for immunohistochemistry up to the secondary antibody incubation, with omission of the endogenous peroxidase inhibition (3% H_2O_2 in methanol for 20 min). Secondary antibody incubations with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (5 µg/mL) or anti-rabbit (1:75) IgG (Zymed) diluted in blocking solution were carried out for 1 hour in the dark. The slides were then washed three times with PBS-T (0.02% Tween 20) and mounted with a drop of mounting media (DAKO). The slides were examined under a laser scanning confocal microscope or fluorescence microscopy under blue excitation.

In-situ hybridization

Small pieces of first trimester placenta were microdissected and verified under a dissecting microscope for the presence of anchoring villous cell columns. Samples were fixed in 4% paraformaldehyde in PBS for 4 hours at room temperature, washed three times in PBS, and dehydrated through a gradient of methanol concentrations up to 100%. Pre-hybridization incubation was carried out for 2 hours at 60°C in hybridization buffer (50% formamide, 0.75M NaCl, 1X PE, 100 μg/mL tRNA, 0.05% heparin, 0.1% BSA, 1% SDS, 05 mg/mL salmon sperm DNA). Probes were prepared from plasmids containing connexin gene PCR fragments in pBluescript. Plasmids were linearized with appropriate restriction enzymes at both 5' and 3' positions in order to label both sense and anti-sense strands. Probes were labeled by *in vitro* transcription in a reaction containing DIG-labeled d-UTP. Labeling reactions contained 2 μg of linearized template, 1X transcription buffer, 1X DIG-RNA labeling mix (Boehringer Mannheim), 0.25 mmol DTT, 40U placental RNase inhibitor (Boehringer Mannheim), and 40U of T3 or T7 RNA polymerase (Boehringer Mannheim) in a 20 μL reaction. After 2 hours of *in vitro* transcription labeling of probes at 37°C, reactions were precipitated with 2μL of 0.2M EDTA, 2.5μL of 4M LiCl, and 75 μL ice-cold 100% ethanol for 30 min at -70°C. Pellets were washed with 70% ethanol and resuspended in 20μL of DEPC-treated ddH_2O. To verify quality and quantity of labeled probe, 1μL was run on a 1% agarose/TAE gel. Placental tissue samples were hybridized with both sense and anti-sense DIG-labeled probes diluted appropriately in hybridization buffer (1.0 – 2.0 μg/mL) overnight at 60°C. Post-hybridization washes were performed as follows: 3 washes with post-hyb wash 1 (0.3M NaCl, 1X PE, 1% SDS) at 63°C, 3 washes with post-hyb wash 2 (50% formamide, 0.3M NaCl, 1X PE, 1.2% SDS) at 63°C, and 3 washes with post-hyb wash 3 (50% formamide, 0.15M NaCl, 1X PE, 0.1% Tween-20) at 50°C. Hybridized probes were detected by immunohistochemistry. Samples were blocked in 2mM levamisole and 10% goat serum in TBST for 1 hour with gentle rocking. Samples were then incubated with anti-DIG-alkaline phosphatase Ab (Boehringer Mannheim) diluted 1:5000 in blocking solution overnight at 4°C, washed 6 times with TBST containing 0.5 mg/mL levamisole, and finally washed 3 times in freshly prepared NTMT (0.1M NaCl, 0.1M Tris pH 9.5, 0.2M $MgCl_2$, 0.01% Tween-20). Colour reactions were performed by incubation of samples with 4.5 μL/mL NBT and 3.5 μL/mL BCIP (NBT/BCIP Combo, GibcoBRL) in NTMT for 5 to

20 minutes to yield a blue colour. Samples were counterstained with hematoxylin for 5 minutes and photographed by digital image capture software (CoolSnap for Mac) under a dissecting microscope.

Statistical Analysis

Semi-quantitative RT-PCR results were analyzed using SigmaStat software (Jandel Scientific). When data approximated normal distribution, parametric statistical analyses were performed. Data were subjected to two-way repeated measures analysis of variance (ANOVA) followed by all pairwise multiple comparison procedures (Student-Newman-Keuls method) to determine between-group differences. When data was not normally distributed, non-parametric statistical analyses were performed using the Kruskal-Wallis one way analysis of variance on ranks (for Cx32 and Cx45). The level of significance for comparisons was set at $p<0.05$.

2.3 Results

RT-PCR screening of connexins in the first trimester human placenta

To determine the connexins that may be important in early human placental development, first trimester placentas were screened by RT-PCR for 8 connexins. Positive PCR amplification products were observed for Cx32, Cx37, Cx40, Cx43 and Cx45 in the first trimester human placenta (Figure 2.1). Each PCR product was of the predicted length and displayed identical mobility to the amplification product in the control tissue. On the other hand, Cx26 and Cx31 transcripts were weakly or not at all detected in the first trimester (Figure 2.2, a and b), although an amplicon of the appropriate size was electropheresed in control tissue PCR reactions (Figure 2.2, c). PCR reactions with Cx46 primers revealed multiple bands, one of which was at the predicted size (Figure 2.2, d). The presence of multiple bands was most likely due to the fact that these primers were designed against rat Cx46, which is known to have high homology to other connexin subtypes. Because of these multiple bands, semi-quantitative RT-PCR was not performed for Cx46, as different mRNA species would compete for amplification, thus making the reaction unquantifiable. In all cases, no amplification product was observed in control reactions in which RNA or water were used as template instead of RT.

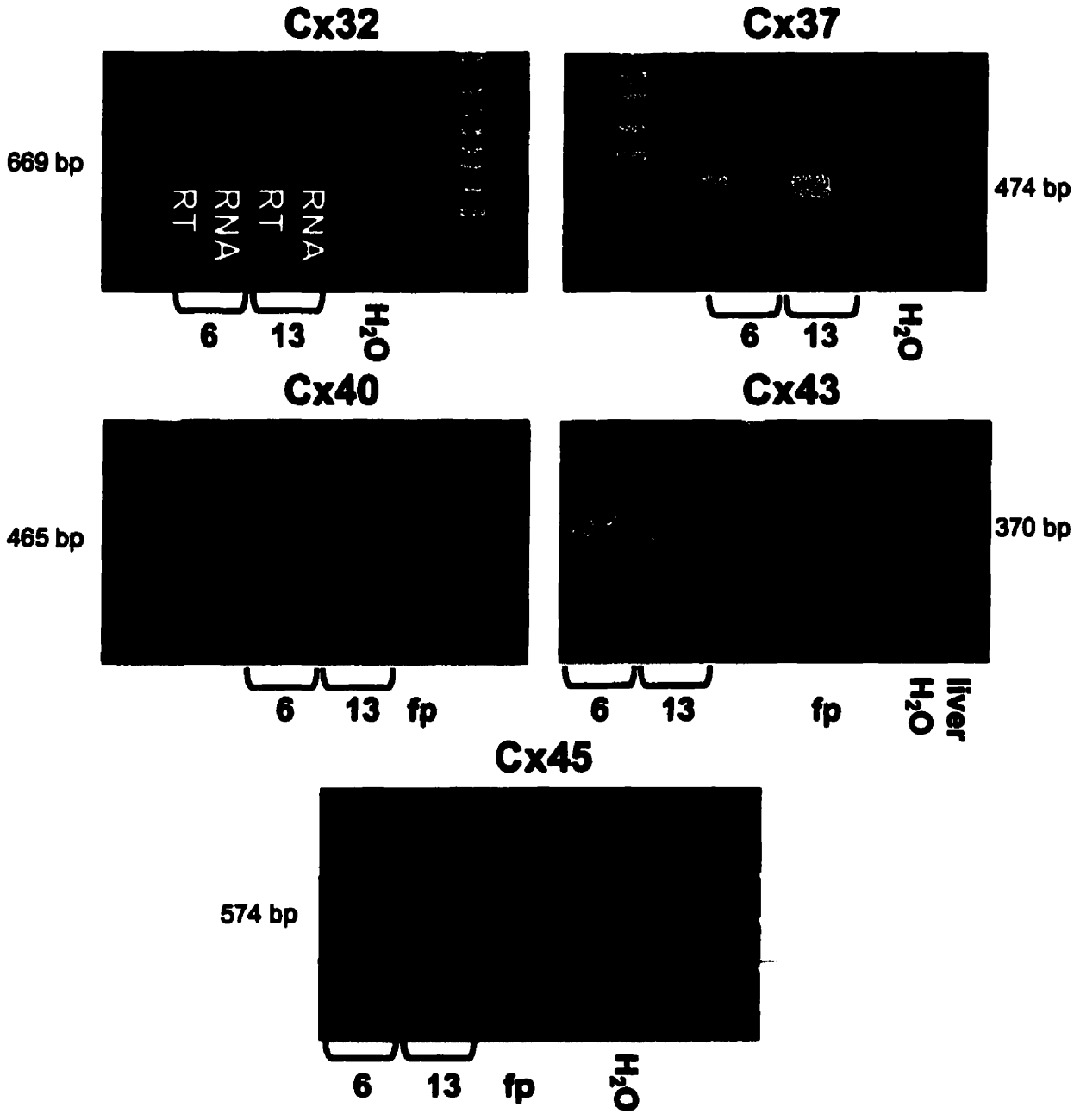

Figure 2.1. Cx32, Cx37, Cx40, Cx43 and Cx45 transcripts are expressed in the early human placenta. RT-PCR amplification products are detectable for Cx32, Cx37, Cx40, Cx43 and Cx45 in 6 week and 13 week human placentas. To verify that DNA contamination was not present in placental RNA, RNA was used in the place of RT as template for PCR amplification (in each case, gels are loaded with RT used as template and then RNA used as template). As a negative control, water was used in place of RT as template. As a positive control, fetal parts (fp) were used.

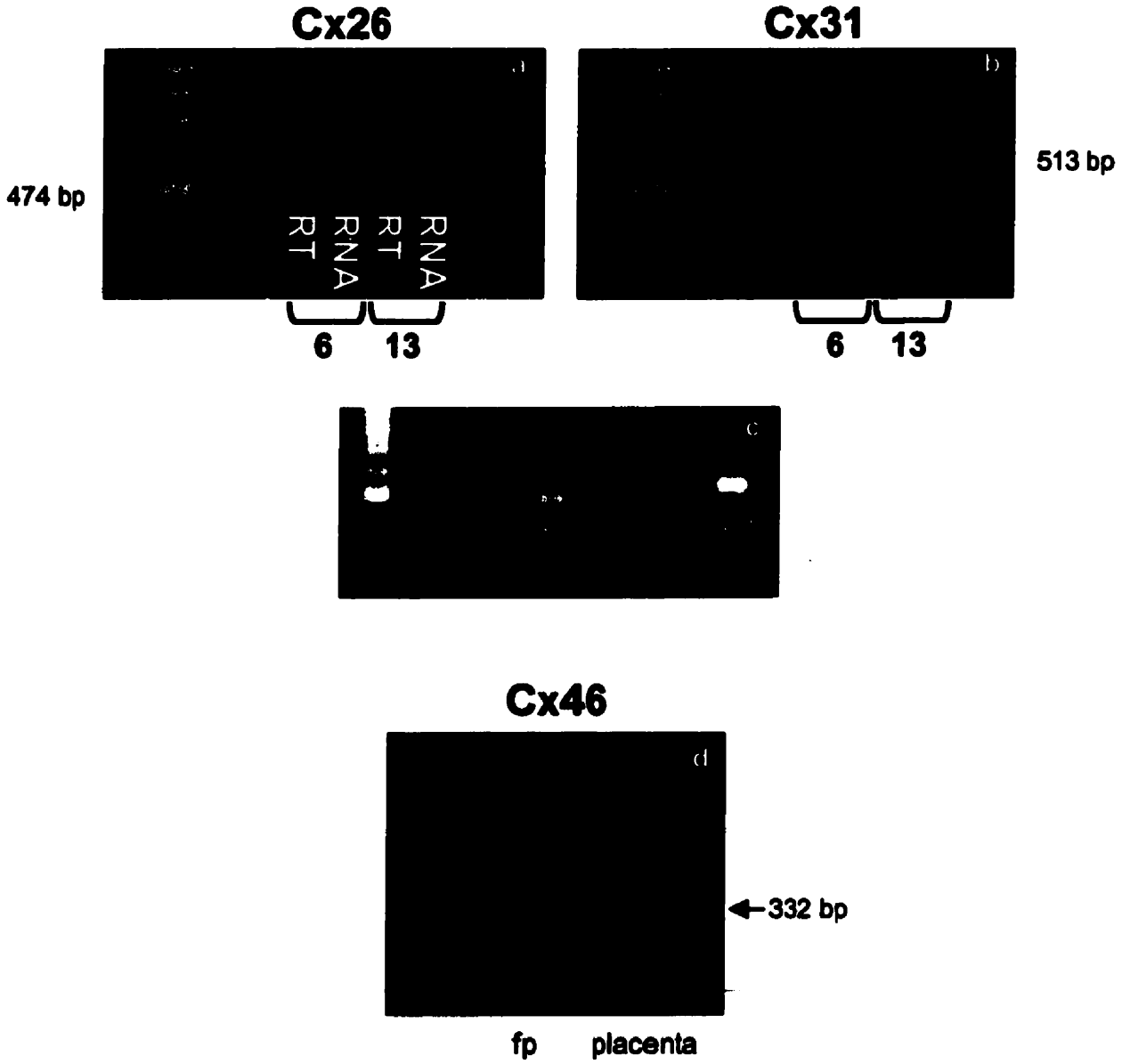

Figure 2.2. Cx26 and Cx31 transcripts are weakly expressed in the early human placenta. (a and b) RT-PCR reactions were carried out using Cx26 or Cx31 primers and either placental RT or RNA (to verify the absence of DNA contamination) samples as template. Amplification products were obtained in control tissues (panel c), but not in the 6 week placenta and very weakly in the 13 week placenta. (B) Cx46 primers, which were designed against the rat Cx46 sequence, amplified many products by RT-PCR, including a band at the predicted 332 bp in placental samples. (fp=fetal tissue, positive control in all cases)

Semi-quantitative connexin RT-PCR throughout the first trimester

Semi-quantitative RT-PCR was performed for Cx32, Cx37, Cx40, Cx43 and Cx45 in order to determine changes in these transcripts over the first trimester of pregnancy. In all samples, RNA was used as template in a PCR reaction using β-actin primers, which span an intron. DNA contamination of RNA would be detected by the amplification of a genomic β-actin band at 359 bp, instead of the transcript size of 265 bp. No band at 359 bp was detected for any RNA sample. First trimester profiles for each connexin were obtained by standardizing the connexin PCR product ROD of each placental sample to its corresponding β-actin ROD, as described in Materials and Methods. Placental RT samples were obtained from at least 3 placentas in each gestational week for statistical analysis.

Cx32: Semi-quantitative RT-PCR revealed that Cx32 mRNA is expressed in very early placentas (6 weeks gestation) and abruptly decreases between 7-12 weeks gestation. At 13 and 14 weeks, Cx32 transcripts are again detectable, at half the abundance as 6-week placentas (Figure 2.3, A). Statistical analysis indicates that Cx32 transcript levels at 6 weeks are significantly higher than at all other time points in the first trimester (Figure 2.4, A).

Cx37: Cx37 mRNA increases throughout the first trimester in the human placenta (Figure 2.3, B). Prior to 10 weeks gestation, transcript levels are relatively low and constant and then mRNA expression is significantly higher between 10 and 14 weeks gestation (Figure 2.4, B).

Cx40: The first trimester profile for Cx40 is similar to that of Cx37 (Figure 2.3, C). Transcript levels are low during the first half, and significantly increase at between 10 and 14 weeks (Figure 2.4, C).

Cx43: Cx43 transcript levels remain constant throughout the first trimester in the human placenta (Figure 2.3, D), with no significant changes at any age (Figure 2.4, D).

Cx45: The profile of Cx45 in the first trimester placenta is similar to Cx37 and Cx40, with low mRNA levels early in the first trimester increasing towards the end of the first trimester (Figure 2.3, E). In the case of Cx45, there is a significant increase in mRNA expression at 14 weeks (Figure 2.4, E).

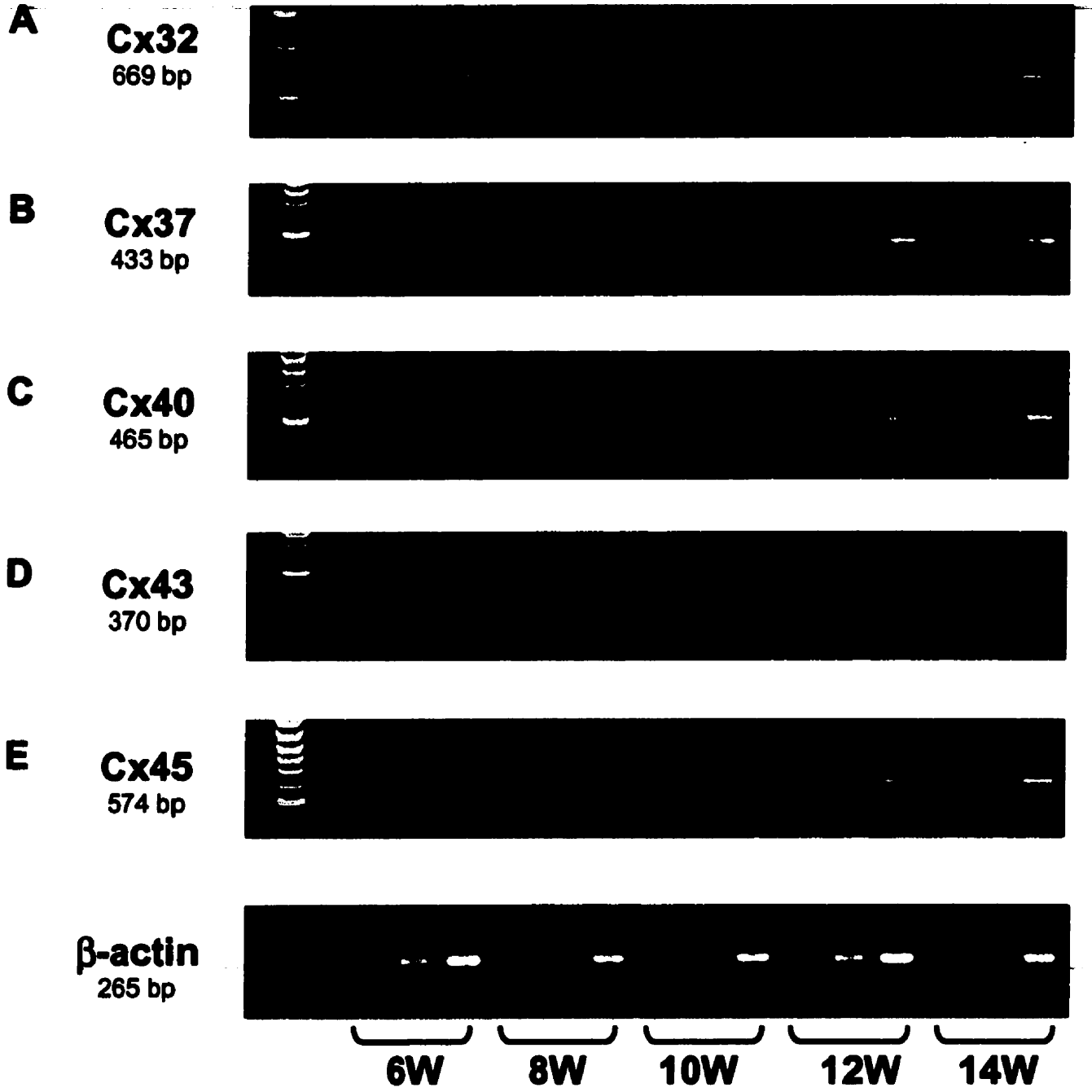

Figure 2.3. Connexin mRNA expression profiles throughout the first trimester in the human placenta. Representative agarose gels showing RT-PCR products for each connexin primer pair and β-actin. For each sample, PCR was performed at 3 cycle numbers in the linear range of each primer pair. These were cycles 26, 28, & 30 for Cx32; 26, 28 & 30 for Cx37; 22, 24, & 26 for Cx40; 23, 25 & 27 for Cx43; 26, 28 & 30 for Cx45; and 17, 19 & 21 cycles for β-actin.

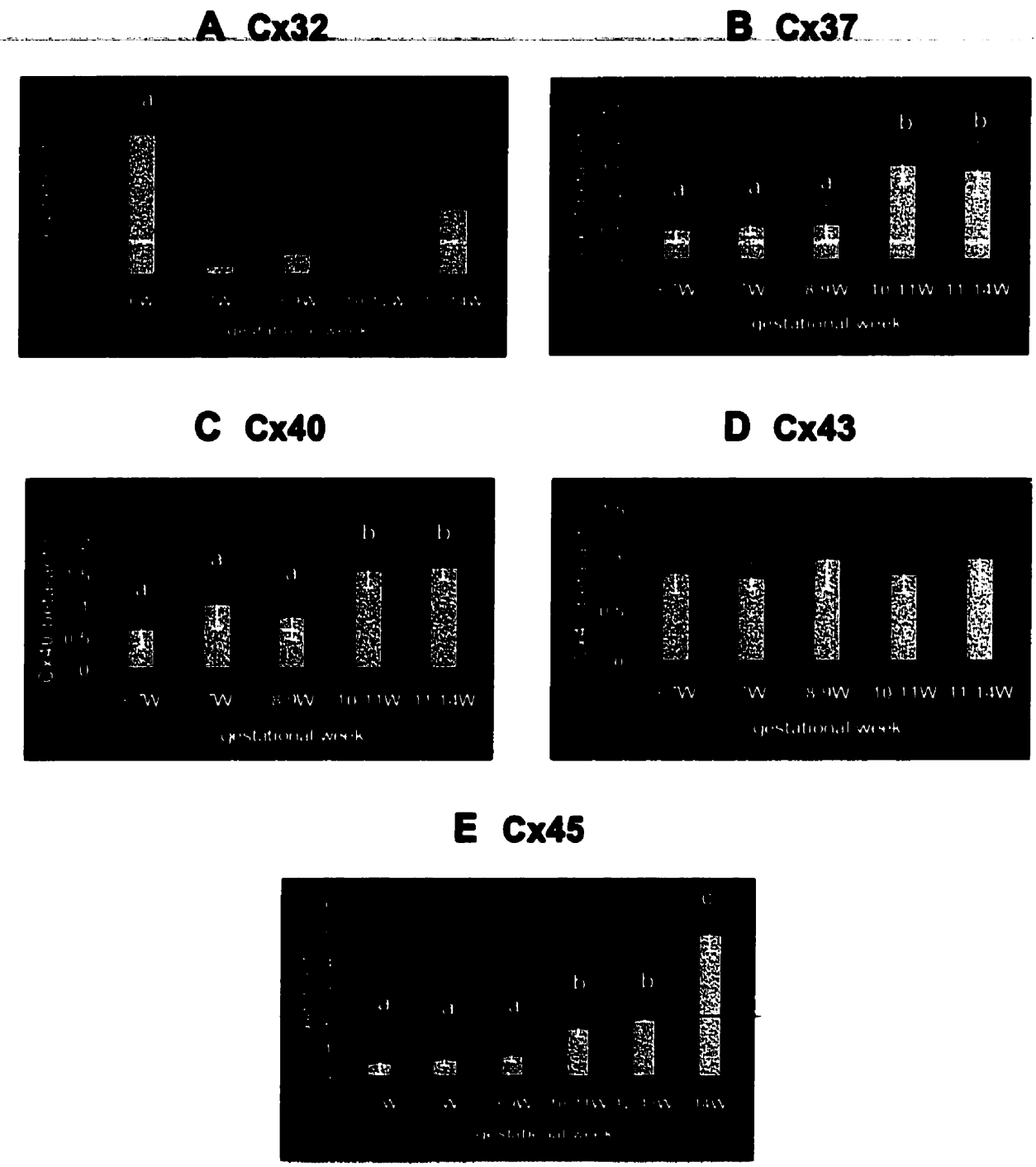

Figure 2.4. Graphs of changes in Cx32, Cx37, Cx40, Cx43 and Cx45 mRNA in the human placenta throughout the first trimester of pregnancy. Graphs showing the mean +/- SEM (n=at least 3 for each group) of the ratio of ROD of each connexin to its corresponding β-actin internal standard. Data labeled with different letters are significantly different from each other ($P<0.05$).

Localization of connexin protein or mRNA in the first trimester human placenta

Connexins 32, 40, 43 and 45 were localized by immunohistochemistry, immunofluorescence, or *in situ* hybridization in first trimester human placental samples. By immunofluorescence, Cx32 protein was immunolocalized at the interface of villous CTB and STB, as well as at locations of villous sprout formation in 6 week placental sections (Figure 2.5, a and b). In 7 to 13 week gestation placentas, no Cx32 staining was evident (data not shown). Punctate staining for Cx40 was evident in all anchoring EVT cell columns and in endothelial cells surrounding villous fetal vessels (Figure 2.5, c and d) at every gestational age throughout the first trimester. Cx43 was immunolocalized only in villous mesenchymal cells throughout the first trimester of pregnancy (Figure 2.5, e). No reactivity was observed in slides stained only with secondary antibody (Figure 2.5, f).

By immunohistochemistry, the expression pattern of Cx43 was confirmed. Cx43 was expressed in villous mesenchymal cells and in endothelial cells lining fetal villous vessels (Figure 2.6, A), and not in EVT cell columns (Figure 2.6, B). Cx45, on the other hand, was detected in anchoring EVT cell columns (identified by HLA-G immunostaining) and in the villous mesenchyme. Cx45 displayed a unique temporal pattern of expression, in that prior to 9 weeks gestation it co-localized with HLA-G in EVT columns (Figure 2.6, C and D), but after 9 weeks expression was lost from this population (Figure 2.6, E and F). However, Cx45 expression increases in the stroma of villi between 7 and 14 weeks gestation.

In situ hybridization was performed by whole-mount techniques. This was done to verify the expression of Cx40 in anchoring EVT cell columns. Figure 2.7 shows the presence of Cx40 transcripts specifically in anchoring EVT cell columns (A) and in villous fetal vessels (B), consistent with Cx40 protein localization. As a control, liver was used to verify the absence of probe hybridization in hepatocytes, whereas endothelial cells around hepatic vessels showed positive staining (Figure 2.7, C). In whole-mount *in situ* hybridization experiments using the DIG-labeled sense probe, no positive staining was detected (data not shown).

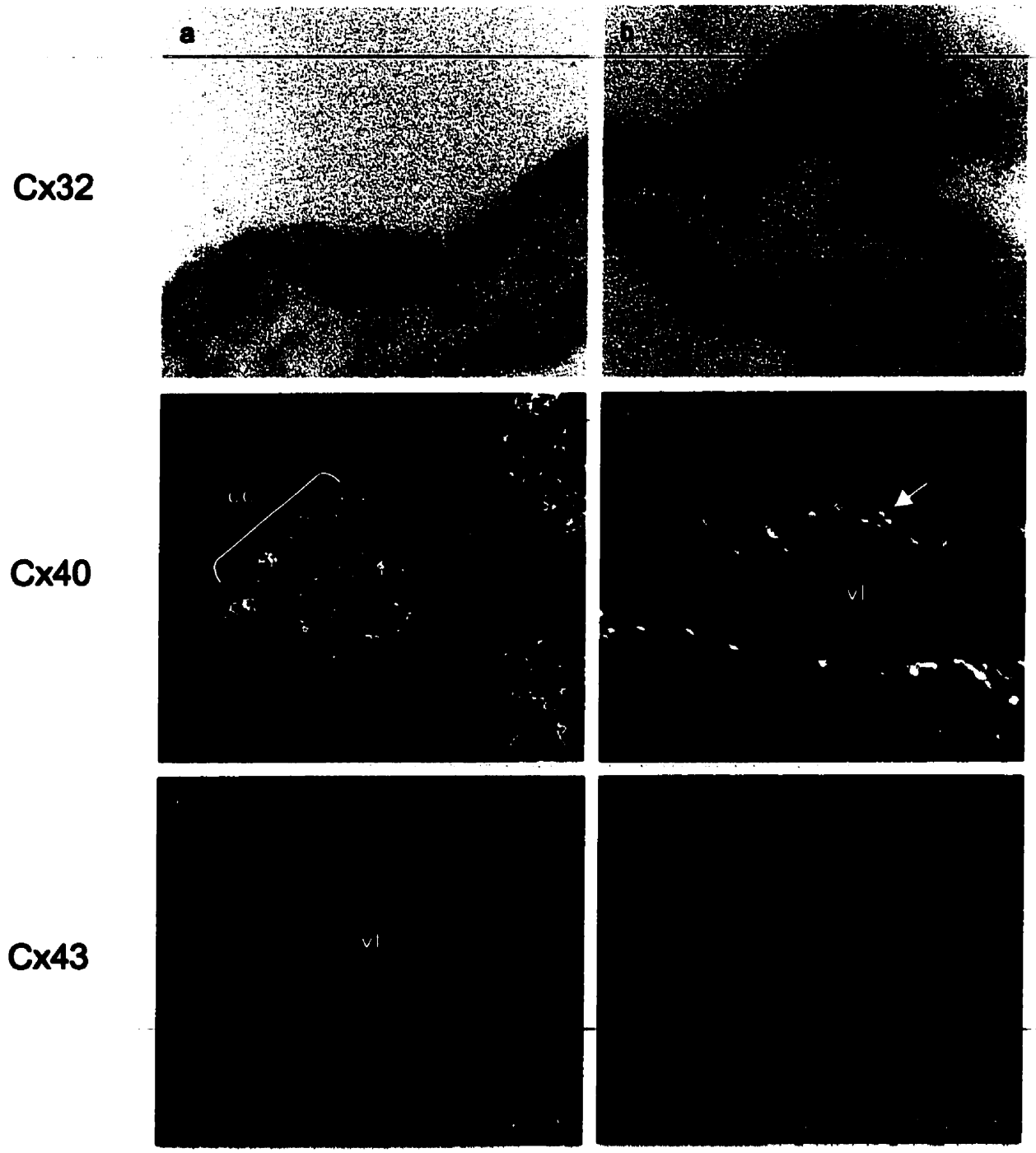

Figure 2.5. Immunofluorescent localization of Cx32, Cx40 and Cx43 in the early human placenta. (a) Cx32 is expressed at the interface between villous cytotrophoblast and STB in this 6 week placenta (arrow). (b) Cx32 is also expressed at locations of cell sprout formation in the 6 week placenta (arrow). (c) Cx40 is strongly expressed in anchoring EVT cell columns, and in villous fetal vessel endothelium (d). Cx43 is expressed in villous mesenchyme and in villous fetal vessel endothelium (e). No reactivity is detected when primary antibody is omitted (f). (vl=vessel lumen, cc=cell column)

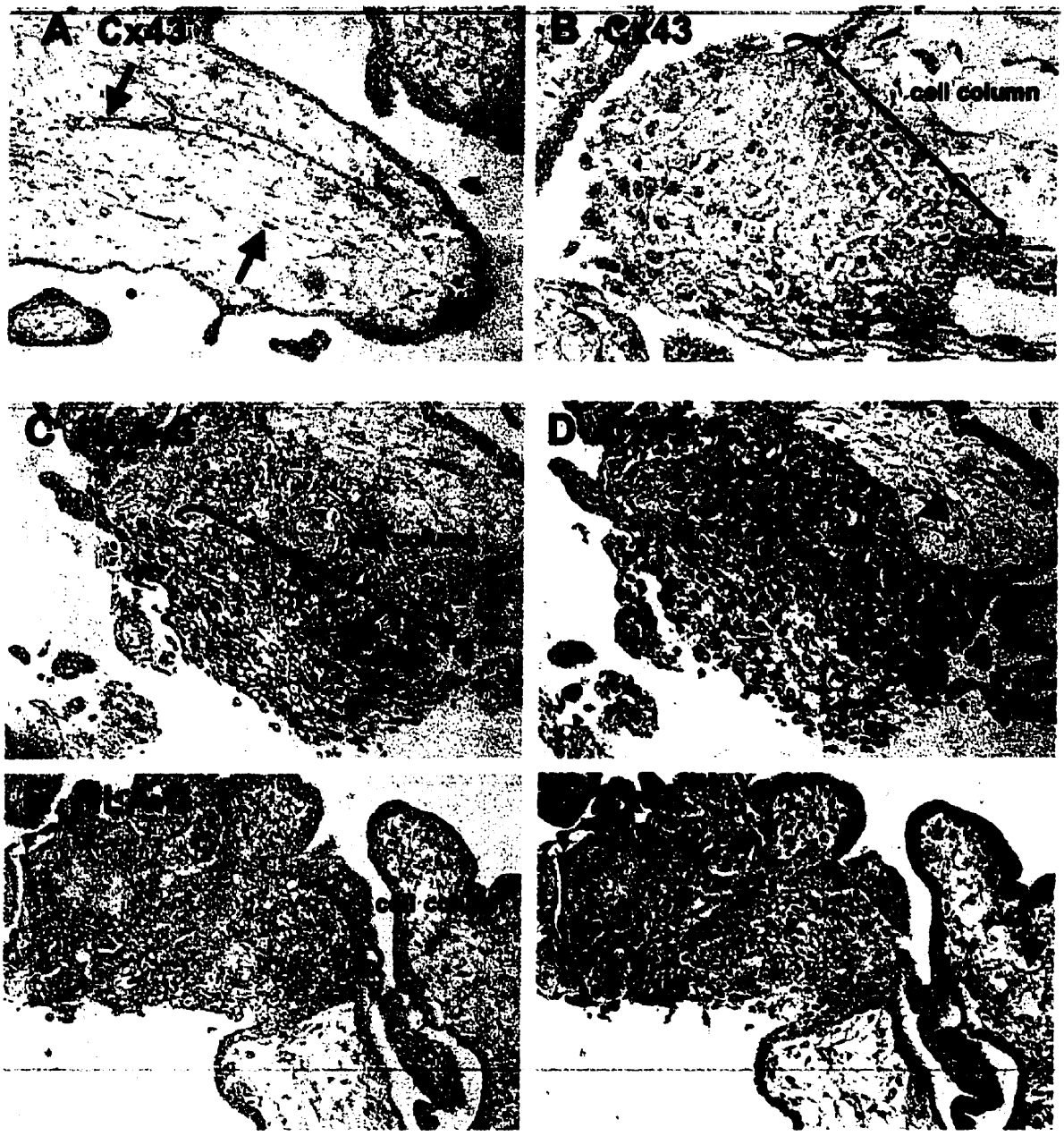

Figure 2.6. Cx43 and Cx45 immunohistochemistry on first trimester placenta sections. (A) Cx43 is expressed in villous mesenchymal tissue and villous vessel endothelial cells in all placentas (arrows, 8 week placenta shown). (B) Cx43 is not expressed in EVT cell columns (13 week placenta shown). (C and E) HLA-G staining to specify EVT cell columns in 7 week (C) and 11 week (E) placentas. (D) Cx45 is expressed in cell columns in 7 week placentas. (F) Cx45 is not expressed in cell columns in placenta greater than 10 weeks gestation (11 week placenta shown) Arrowheads in D and F indicate stromal Cx45 staining. Note the blue counterstained stroma in the 7 week placenta compared to the brown (positive) stroma in the 11 week placenta. (magnification=200X)

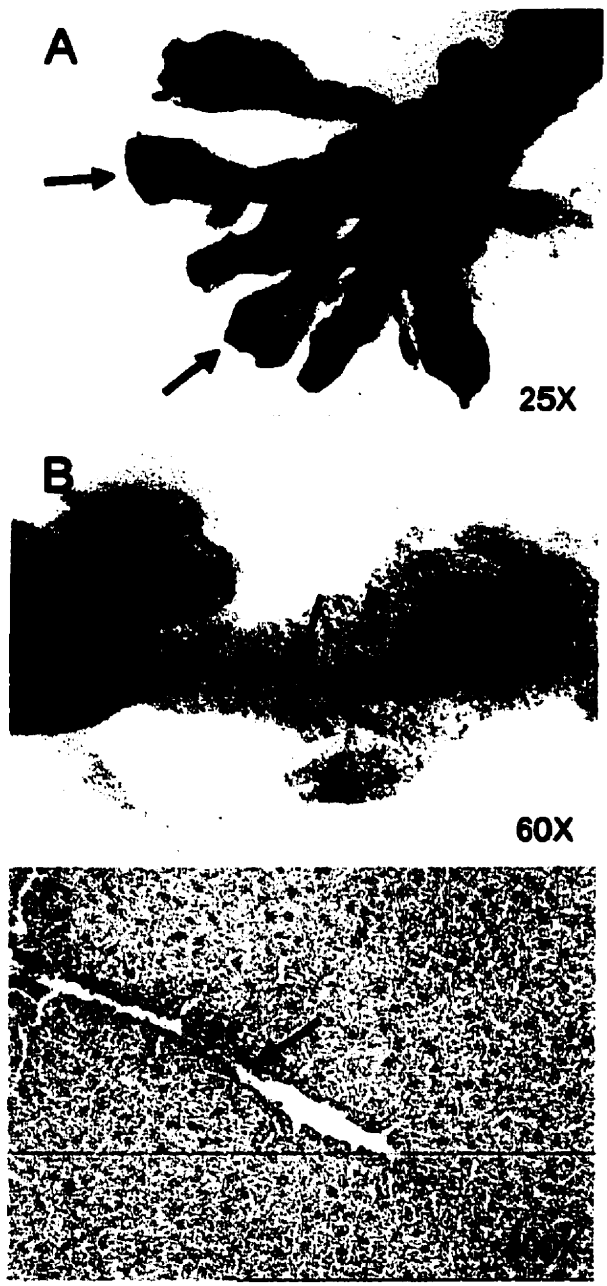

Figure 2.7. Cx40 whole-mount *in situ* hybridization. (A) Cx40 mRNA is expressed specifically in anchoring EVT cell columns (dark blue against pink counterstain, arrows). (B) Cx40 mRNA is also localized in villous fetal vessel endothelial cells (arrow). (C) Control tissue (liver) sectioned after whole-mount staining for Cx40 demonstrating the absence of Cx40 mRNA in hepatocytes but expression of Cx40 (blue colour against pink counterstain) in endothelial cells surrounding a hepatic vessel (arrow).

2.4 Discussion

In this study, connexin expression in the early human placenta was assessed using RT-PCR and immunlocalization techniques. By RT-PCR, the following connexins were identified in pooled first trimester cDNA – Cx32, Cx37, Cx40, Cx43 and Cx45. In other cell types, each of these has been shown to form functional channels that vary widely in permeability, conductance, gating, and other properties (222). Following transcript levels by semi-quantitative RT-PCR, these connexins were found to exhibit changes in expression across the first trimester, with the exception of Cx43, which remained constant during this period. Furthermore, the connexins are expressed in different cell populations at different times throughout the first trimester. Results from the present study indicate that multiple connexins are expressed in the early human placenta, and their patterns of expression suggest various roles for each connexin subtype during placentation.

In the PCR studies, each connexin primer sequence was verified for specificity by screening all nucleotide sequences on GenBank (NCBI web-site). Amplified products were verified for identity by sequencing. For the semi-quantitative RT-PCR results presented in this study, therefore, each primer pair amplified only the specific connexin it was designed against. The main limitation of the RT-PCR protocol is the heterogeneity of tissue samples used to extract RNA. The human placenta is composed of smooth chorion, chorionic villi, and EVT cell columns. In some samples, many villi are damaged and separated from the placenta during the operative procedure, resulting in a proportionally greater amount of smooth chorion. Similarly, in some samples there are very few EVT cell columns remaining at the tips of villi, whereas in others there are many cell columns intact. With the immunolocalization experiments, antibodies were not pre-absorbed with peptide to ensure specificity, since peptides were not available. The group that raised most of the connexin antibodies (all but the Cx43) used in this study (Otto Traub, Germany) has produced antibodies for most connexins identified to date. They are interested in tissue-specific expressions of connexins, and their antibodies have been validated for specificity (223, 224).

Cx32 transcript levels were high in 6-week placentas and then levels declined between 7 and 12 weeks in all but one placenta sample. At 13 and 14 weeks, Cx32 mRNA was upregulated once more, to approximately half the 6-week levels. Cx32 was immunolocalized in 6-week

placentas to the area between villous CTBs and STB, as well as to EVT cell sprouts. Because of the preponderance of Cx32 in young placentas (less than 7 weeks) and its specific expression pattern, it is possible that this connexin is important in early trophoblast syncytialization and early villous branching morphogenesis. Gap junctions are known to be important in mediating cell fusion events and recently it has been reported that Cx43 is expressed during both *in vivo* and *in vitro* trophoblast syncytialization (216). From the results of the present study, Cx32 may also be involved in this process, since it is expressed at the interface of villous CTB and syncytium, where GJIC is critical for differentiation of CTB stem cells to syncytium (225). The presence of GJIC communication between these two cell populations is thought to provide a 'physiological syncytium,' allowing the exchange of ions and small molecules, preceding the morphological fusion into syncytium (215). During the *in vitro* process of trophoblast syncytialization, Cronier *et al.* have detected Cx43 (but not Cx32) prior to fusion (226), but they utilized trophoblast cells isolated from term placentas in their experiments. It is possible that trophoblasts isolated from 6-week placentas would continue to express Cx32 in culture, and that this connexin could then contribute to differentiation towards syncytium. Interestingly, aggregated EVT trophoblast cells in the maternal decidua, which are thought to undergo fusion to form multinucleated trophoblast giant cells, also express Cx32 (as well as Cx43) (30). This provides further support for a role of Cx32 in trophoblast cell fusion, either towards villous STB or towards placental bed trophoblast giant cells.

Transcript levels of Cx37, Cx40, and Cx45 increased during the first trimester. Because of the localization of Cx37 and Cx40 to vascular endothelial cells ((213) and the present study), the increase in these two connexins most likely reflects the considerable vasculogenesis and angiogenesis that occurs in placental villi during this period. From immunohistochemical localization of Cx45, it would appear that the increase in Cx45 mRNA is preserved at the protein level by an increase in villous mesenchymal expression. Cx45 is also expressed in fibroblast cell populations in the small intestine (227), in the adult lung, and in smooth muscle cells of the heart, as well as in embryonic brain, skin, and kidney. Expression of Cx45 in these tissues is approximately 40-fold greater at the embryonic stage than in the corresponding adult tissue (228). In this light, Cx45 may have a particular function in developing systems that is not required in adult tissues, and the presence of Cx45 in placental villous mesenchymal tissue may be due to this unique embryonic role. What this specialized role is remains to be determined.

Cx43 protein was also localized to villous mesenchyme through all stages in the first trimester. Co-expression of Cx43 and Cx45 has been observed by immunofluorescent microscopy between granulosa cells in ovarian tissue (229), and in the heart (230). However, the functional significance of this combination of connexins in these tissues and in placental villous mesenchyme is unknown. Co-expression of connexins is common in many tissues, but there are no clear patterns as to which connexins are co-expressed and what the purpose of these combinations is.

Expression of Cx40 and Cx45 protein was detected by immunostaining in anchoring cell columns in the first trimester human placenta. Cx40 was also found to be restricted in this cell population by Winterhager *et al.*(213), but they did not assess Cx45 expression. Winterhager *et al.* also detected Cx40 immunoreactivity in trophoblast aggregates in the placental bed, where Cx32 and Cx43 are also expressed, but not in non-aggregated, invading EVT cells in the decidua. Therefore, Cx40 appears to be an EVT specific connexin that is only expressed when trophoblast cells are in contact with other trophoblast cells, and not with other cell-types (eg. decidual cells). This is supported by presence of Cx40 expression in choriocarcinoma cell lines (BeWo and JAr cells) (214), as well as in isolated trophoblast cells from first and second trimester placentas (231). The model arising from these observations is that Cx40 is required for proliferative, migrating EVT cell columns, but expression is lost in individual, invading trophoblast cells in the decidua. Winterhager *et al.* propose that Cx40 is upregulated again once EVT cells reach maternal vessels and replace endothelial cells surrounding these vessels. Since endothelial cells are known to express Cx40, this is in accord with the model put forward by Zhou and Damsky, in which EVT cells are thought to adopt a vascular phenotype as they approach maternal vessels in the decidua (117). Several characteristics of endothelial cells are recapitulated in trophoblast cells once they have invaded into the decidua and appose maternal blood vessels. For example, they upregulate VE-cadherin and $\alpha 1\beta 1$ integrin, both of which are expressed on endothelial cells surrounding maternal vessels.

Cx45 expression in EVT cell columns is much weaker than Cx40, and is down-regulated after 9 weeks gestation in this location. As mentioned earlier, Cx45 is expressed in many embryonic tissues, but in the corresponding adult tissue expression is greatly reduced. During mouse heart development, Cx45 is expressed between days 8.5 and 10.5 post-coitum (pc), and is then down-regulated from day 11 pc onward, and in the major part of the adult heart, Cx45 is

undetectable (232). Together, these data raise the possibility that Cx45 may be regulated by oxygen tension in some tissues. Embryonic tissues are exposed to a much lower oxygen tension than adult tissues, and the oxygen tension in the human placenta prior to 9 weeks gestation is much less than after 9 weeks (18 mmHg compared to 60 mmHg) (125). It has been proposed that other genes are regulated in this manner in the early placenta, such as TGF-β3 (134). Caniggia *et al.* have shown in first trimester villous explants that low oxygen (3%) induces the expression of both HIF-1α mRNA and TGF-β3 mRNA. Furthermore, when they 'knock-down' HIF-1α protein in explants grown in 3% O_2 by addition of anti-sense oligonucleotides to HIF-1α mRNA, TGF-β3 expression also decreases. They have also shown *in situ* that TGF-β3 levels change during the first trimester in the human placenta: expression is high prior to 9 weeks gestation and falls thereafter. Based on these results, it could hold true that Cx45 is also regulated by HIF-1, and that in the low oxygen environment prior to 9 weeks gestation levels are high in anchoring column EVT cells, whereas after this time expression decreases. This is paradoxical to the increase in transcript abundance of Cx45 determined by RT-PCR in the present study. This might be explained by cell-type specific regulation of Cx45 transcription. The majority of Cx45 is expressed in villous mesenchymal tissue, which may not possess the same transcriptional regulatory mechanisms as trophoblast cells. In the villous stroma, Cx45 expression increases with increasing gestational age, indicating that in this cell-type oxygen tension does not affect transcription.

From this study, it is clear that connexin expression during human placental development is yet another example of the complex patterns of connexins observed in tissues during development. For example, in developing rat motor neurons, Cx36, Cx37, Cx40, Cx43, and Cx45 are expressed prenatally, but Cx40 and Cx45 disappear following birth (233). The significance of such regulation of connexin expression during motor neuron development is not well understood. The results of the present study suggest multiple roles for 5 connexins in the developing human placenta. A known role for gap junctions in the rodent placenta is in the transfer of glucose between the two STB layers of the placenta (211). Since the human placenta is not structured in this manner, gap junctions are not required for this specific purpose. Instead, they seem to mediate fusion of stem villous CTBs to syncytium (Cx32), vessel function (Cx37 and Cx40), villous mesenchyme structure (Cx43 and Cx45), and EVT cell column function (Cx40 and Cx45). In particular, Cx40 is abundant in EVT cell columns throughout the first

trimester, and expression is lost once the cells break away from the column and invade the decidua individually (213). A down-regulation of Cx40 may be important to allow EVT at the distal edge of cell columns to break away and invade, since decreased GJIC correlates with increased invasive capacity of tumour cells as well. To further investigate EVT cell differentiation and invasion, a placental villous explant culture system could be used, since villous explants cultured at 3% O_2 undergo proliferation of trophoblasts at villous tips to form outgrowths of EVT cells that are analogous to cell columns. Using this system, GJIC could be blocked and changes in the phenotype of the EVT cells could be assessed. These experiments will be discussed in detail in the following chapter.

CHAPTER 3

THE ROLE OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN HUMAN PLACENTAL VILLOUS EXPLANT CULTURES

3.1 Introduction

Gap junctions have been identified in both the developing rodent and human placenta-. Human placental development requires the establishment of a proliferating group of trophoblast cells at the tips of anchoring villi in order to secure the placenta to the wall of the decidua. Furthermore, EVT cells emanating from these anchoring cell columns undergo a differentiation program in which they become highly invasive, invading into the maternal decidua to replace the endothelial cells surrounding maternal blood vessels. This process results in the dilation of maternal vessels, which increases blood flow through the placenta to the developing embryo. Human EVT cell differentiation has been extensively studied, and numerous proteins are known to be important during this process, including transcription factors (13), growth factors/cytokines (3), matrix metalloproteinases (9), and integrins and cadherins (117).

In Chapter 2, we investigated gap junction expression in the early human placenta, since gap junctions are known to mediate cell proliferation, migration and invasion, all of which are required for many developmental processes, including neural crest cell migration (171), myogenesis (234), and possibly human placentation. Five gap junction proteins (connexins) were identified in the first trimester human placenta. Two of these, Cx40 and Cx45, were localized to anchoring EVT cell columns in early placental samples. In particular, Cx40 expression was very prominent, and was maintained throughout the first trimester in anchoring columns. Because of the presence of connexins in anchoring villous columns, we sought to verify a requirement for gap junctional communication in cell columns using an *in vitro* placental villous explant system. When first trimester human placental villous explants are cultured in low oxygen (3% O_2) on Matrigel substrate, trophoblast cells at the tips of villi undergo proliferation to form an outgrowth of EVT cells (11). These express phenotypic markers characteristic of EVT cell columns *in vivo*, such as the proliferation marker Ki67, matrix-metalloproteinase-2 (MMP-2), $\alpha5\beta1$ integrin, and E-cadherin (235).

In the present study, the role of GJIC in explant EVT outgrowths was investigated by treating placental villous explants (dissected from 6-8 week gestation placentas) with three classes of GJIC uncoupling agents; the glycyrrhetinic acid derivative carbenoxolone (CBX, 125 μM), the volatile alcohol heptanol (5 mM), and the endogenous N-acylethanolamine oleamide/sleepamide (50 μM). Glycyrrhetinic acid derivatives are potent inhibitors of gap

junction function and have been proposed to interact directly with connexins to perturb gap junctions (236). Carbenoxolone is also known to inhibit 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2), an enzyme present in STB that converts cortisol to cortisone. Volatile agents such as octanol and heptanol are thought to inhibit GJIC indirectly by altering the hydrophobic environment of the plasma membrane (237). Oleamide is an endogenous fatty acid that accumulates in cerebrospinal fluid under conditions of sleep deprivation (238), and is a less potent uncoupling agent (239). CBX was tested for its ability to inhibit GJIC in a trophoblast cell line (JAr) by scrape-loading methods, and in explant trophoblast outgrowths by micro-injection techniques. The phenotype of outgrowth EVT cells following GJIC-blockade was characterized by analyzing expression of Ki67, HLA-G (an EVT-specific marker), α5β1 and α1β1 integrins, and MMP-2 activity by gelatin zymography.

Since Cx40 is the predominant connexin in anchoring cell columns throughout the first trimester of pregnancy, we specifically down-regulated Cx40 expression in placental villous explant cultures using phosphorothioated anti-sense oligonucleotides generated towards the sequence flanking the translation start site of Cx40 mRNA. Such a strategy has previously been used successfully in placental villous explant cultures to 'knock-down' the protein expression of TGF-β3 and HIF-1α (88). Cx40 is expressed in anchoring trophoblast columns both *in vivo* and *in vitro*, but expression is down-regulated *in vivo* in individual EVT cells that have differentiated into the invasive phenotype. The specific targeting of Cx40 protein in this study by anti-sense oligonucleotides would address the role of the down-regulation of Cx40 *in vivo*. Explants were treated with anti-sense oligonucleotides to Cx40 mRNA and changes in outgrowth EVT morphology were assessed.

3.2 Materials and Methods

Human placental villous explant culture

Villous explant cultures were established from first trimester human placentas by a modification of the method of Genbachev *et al.* (31). First trimester human placentas (6-8 weeks gestation) were obtained from elective terminations of pregnancies by dilatation and curettage. Informed consent was obtained from each patient and collections were approved by the

University of Toronto's Review Committee on the Use of Human Subjects. Placental tissue was placed in ice-cold PBS and processed within 2 hours of collection. The tissue was washed in sterile PBS and small fragments of placental villi (15-20 mg wet wt) were dissected from the placenta, teased apart, and placed on Millicell-CM culture dish inserts (pore size 0.4 μm, Millipore Corp) precoated with 0.2 mL undiluted Matrigel substrate (Collaborative Research). The Matrigel was allowed to polymerize at 37°C for 30 min. Placental villous explants were cultured in serum-free DMEM-Ham's F-12 media (Life Technologies) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.25 μg/mL ascorbic acid, pH 7.4 at 3% O_2/5% CO_2. Culture media was changed every 24 hours, and collected for measurement of matrix-metalloproteinases. Villous explants were maintained in culture for up to 6 days. Flattening of the distal end of the villous tips, their adherence to Matrigel, and the appearance of EVT breaking through from the tips were used as markers of viability and morphologic integrity as previously described by Genbachev *et al.* (31). Explants (from a single placenta) were cultured in triplicate for each treatment point. Each experiment was repeated with at least 3 placentas.

GJIC inhibitor and antisense oligonucleotide treatments of explants

Three gap junctional communication inhibitors were used in this study; carbenoxolone (CBX) (125 μM, Sigma), heptanol (2 mM, Sigma) and oleamide (20-100 μM, Sigma). As a control for CBX, glycyrrhizic acid (GZA) (125 μM, Sigma) was also used. This reagent is structurally similar to CBX but does not block GJIC. Stock solutions of CBX and GZA (20 mM) were prepared in sterile distilled water and stored at 4°C. For treatments, reagents were diluted in DMEM-Ham's F-12 media (Life Technologies) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.25 μg/mL ascorbic acid, pH 7.4. Treatments began on either day 1 (the day after dissection) or day 3 and experiments were carried out for up to 6 days. Conditioned media was collected every 24 hours and kept frozen at -20°C for future use (zymography).

Phosphorothioated oligonucleotides were synthesized and purified by capillary electrophoresis (Life Technologies). An oligonucleotide of 18 bp targeted against sequences adjacent to the AUG translation initiation codon of human Cx40 mRNA was synthesized, as well as an oligomer with the same bp composition as the antisense oligonucleotide but with a scrambled sequence. The sequences of the antisense and scrambled Cx40 oligonucleotides were

5'-TACCCGCTAACCTCGAAG-3' and 5'-CATGCCATCCCAGCTGAA-3', respectively. Oligonucleotides were dissolved in water at a concentration of 100 μM. Antisense and scrambled oligonucleotides were diluted 1:10 in culture media to a final concentration of 10 μM and added to the villous explants on day 3 of culture. Explants were monitored and photographed daily for up to 5 days.

Cell Culture

The human choriocarcinoma cell line (JAr, a gift from Dr. Jay Cross, Samuel Lunenfeld Research Institute) was plated on 100mm tissue culture plates and cultured in DMEM high glucose media (Gibco Technologies) supplemented with 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.5% (vol/vol) β-mercaptoethanol under standard conditions. Cells were passaged by trypsinization for 1 min with 0.05% trypsin/0.02% EDTA at 37°C.

Scrape-loading and micro-injection assessment of GJIC

The scrape-loading technique was used to assess the ability of carbenoxolone to inhibit gap junctional communication specifically in trophoblast cells that express high levels of Cx40 (the JAr human choriocarcinoma cell line). The scrape-loading procedure was a modification of that described by El-Fouly *et al.* (240). JAr cells were grown to near confluency in 6-well plates and incubation medium was replaced with fresh media containing CBX (125 μM) or GZA (125 μM) in triplicate for 24 hours. The medium was then removed, the cultures were rinsed 3 times with prewarmed PBS, and 1 mL of a 0.05% solution of Lucifer yellow in PBS was added to each well. The edge of a razor blade was scraped along the monolayer to form the "scrape line" along which the dye enters the cells. After 2 min incubation at room temperature, wells were rinsed 3 times in warm PBS and then incubated in PBS for 10 min to allow dye transfer away from the scrape line. The PBS was then removed and the cultures were fixed in 4% paraformaldehyde in PBS for 15 min. Each culture dish was rinsed 3 times with PBS and 1 mL of 90% (v/v) glycerol was added to each well. The culture dishes were examined under blue excitation (450-490 nM) using a fluorescence microscope (Leica, Leitz DM RXE) and photographed by digital image capture (Openlab software for Mac).

The degree of cell coupling in the presence of carbenoxolone was also assessed in villous explant EVT outgrowths by micro-injection. Explant cultures were allowed to grow for 3 days

to ensure large outgrowth formation. They were treated with CBX (125 μM) or the analog control GZA (125 μM) for 24 hours and then micro-injected with Calcein-AM, a fluorescent dye that must be cleaved by esterases in cells before fluorescence can be detected. Individual EVT cells in villous explant outgrowths were micro-injected with a solution of 1 mM calcein-AM in 150 mM LiCl through glass capillaries using an Eppendorf Transjector (Model 5246). Cells were photographed for 7 minutes after injection using FITC epi-illumination and excitation filters. The time-lapse images were captured by Hamanatsu CCD camera (Model C4742-95) using SimplePCI software (Compix, Inc.). Visual inspection of images was used to assess coupling.

Fluorescein Diacetate-Propidium Iodide Cell Viability Assay

To determine whether treatment of villous explant cultures with CBX (125 μM) causes toxicity to trophoblast cells (JAr cells), the fluorescein diacetate (FDA)-propidium iodide (PI) staining technique was used. FDA, a nonpolar ester, passes through cell membranes and is hydrolized by intracellular esterases to produce fluorescein, which exhibits green fluorescence exclusively in viable cells when excited by blue light. PI only stains injured or dead cells by passing through injured membranes and intercalating with DNA or RNA to form a bright red fluorescent complex. A stock solution of FDA (Sigma) was prepared by dissolving 5 mg/mL in acetone. The FDA working solution was freshly prepared by diluting 40 μL of FDA stock solution in 10 mL PBS. A stock solution of PI was prepared by dissolving 1 mg PI (Sigma) in 50 mL PBS. Confluent JAr cells were treated with either CBX (125 μM) or GZA (control analog, 125 μM) for 24 hours. To stain with FDA-PI, control and CBX-treated JAr cells were first washed twice with PBS. Cells were then covered with 1 mL of PBS, 500 μL of FDA working solution, and 150 μL of PI stock solution and incubated for 3 min. Dyes were removed from the cells and plates were viewed under fluorescent microscopy (Leica, Leitz DM RXE). Images were captured using Northern Eclipse software (version 5.0).

Immunohistochemistry and Immunofluorescence

For immunolocalization experiments, either immunohistochemistry or immunofluorescence techniques were used. Villous explant cultures were harvested and rinsed in ice-cold PBS before fixation in 4% paraformaldehyde for 4 hours at room temperature or overnight at 4°C. The explants were then washed in PBS, and dehydrated for paraffin-

embedding through a gradient series of ethanol in PBS up to 100% ethanol. Samples were cleared in xylene for 1 hour, and infiltrated with paraffin for 4 hours or overnight. Paraffin embedded samples were sectioned at 6 μM using a microtome. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated through a gradient series of ethanol in PBS. Endogenous peroxidase activity was blocked by incubation of the sections in 3% H_2O_2 in methanol for 20 min. Following PBS washes, antigens were unmasked using an optimal antigen retrieval treatment determined for each antibody (0.125% trypsin, 5-10 μg/mL proteinase K, 10 mM sodium citrate, or 0.02-0.2% Triton-X100). Sections were washed 3 times in PBS for 5 min, and nonspecific binding sites were blocked in a solution containing 5% normal goat serum and 1% BSA in PBS at room temperature for 30 min. Primary antibody dilutions are summarized in Table 3.1.

Table 3.1. Primary antibody dilutions.

HLA-G	mono	1:20	gift from Jay Cross, SLRI
Cx40	poly	1:100	gift from Elke Winterhager, University of Essen
Ki67	mono	1:20	Immunotech, Inc.
α1 integrin	mono	1:75	Chemicon International, Inc.
α5 integrin	mono	1:75	Chemicon International, Inc.

Incubations with primary antibodies were carried out at room temperature for 2 hours or overnight at 4°C. Following three 5 min washes with PBS, sections were incubated with biotinylated anti-mouse (1:300) or anti-rabbit (1:750) IgG (Vector Laboratories) diluted in blocking solution for 1 hour at room temperature. After washing 3 times with PBS, slides were incubated with an avidin-biotin complex (Vector Laboratories) for 1.5 hours. Slides were washed again in PBS and developed in 0.075% (wt/vol) 3,3-diaminobenzidine in PBS containing 0.002% (vol/vol) H_2O_2 with or without nickel enhancement (Vector Laboratories), to yield a black product (with nickel enhancement) or a brown product (without nickel enhancement). After light counterstaining with Alcian Blue/Methyl Green (Sigma), slides were dehydrated in an ascending ethanol series, cleared in xylene and mounted with Permount (Fisher). In control experiments, primary antibodies were replaced with blocking solution.

For immunofluorescence, sections were treated following the same steps as for immunohistochemistry up to the secondary antibody incubation, with omission of the endogenous peroxidase inhibition (3% H_2O_2 in methanol for 20 min). Secondary antibody incubations with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (5 µg/mL) or anti-rabbit (1:75) IgG (company) diluted in blocking solution were carried out for 1 hour in the dark. The slides were then washed three times with PBS-T (0.02% Tween 20) and mounted with a drop of mounting media (DAKO). The slides were examined under a laser scanning confocal microscope.

In-situ hybridization

Villous explants were fixed in 4% paraformaldehyde in PBS for 4 hours at room temperature, washed three times in PBS, and dehydrated through a gradient of methanol concentrations up to 100%. Pre-hybridization incubation was carried out for 2 hours at 60°C in hybridization buffer (50% formamide, 0.75M NaCl, 1X PE, 100 µg/mL tRNA, 0.05% heparin, 0.1% BSA, 1% SDS, 05 mg/mL salmon sperm DNA). Probes were prepared from plasmids containing connexin gene PCR fragments in pBluescript. Plasmids were linearized with appropriate restriction enzymes at both 5' and 3' positions in order to label both sense and anti-sense strands. Probes were labeled by *in vitro* transcription in a reaction containing DIG-labeled d-UTP. Labeling reactions contained 2 µg of linearized template, 1X transcription buffer, 1X DIG-RNA labeling mix (Boehringer Mannheim), 0.25 mmol DTT, 40U placental RNase inhibitor (Boehringer Mannheim), and 40U of T3 or T7 RNA polymerase in a 20 µL reaction. After 2 hours of *in vitro* transcription labeling of probes at 37°C, reactions were precipitated with 2µL of 0.2M EDTA, 2.5µL of 4M LiCl, and 75 µL ice-cold 100% ethanol for 30 min at -70°C. Pellets were washed with 70% ethanol and resuspended in 20µL of DEPC-treated ddH_2O. To verify quality and quantity of labeled probe, 1µL was run on a 1% agarose/TAE gel. Explant samples were hybridized with both sense and anti-sense DIG-labeled probes diluted appropriately in hybridization buffer (1.0 – 2.0 µg/mL) overnight at 60°C. Post-hybridization washes were performed as follows: 3 washes with post-hyb wash 1 (0.3M NaCl, 1X PE, 1% SDS) at 63°C, 3 washes with post-hyb wash 2 (50% formamide, 0.3M NaCl, 1X PE, 1.2% SDS) at 63°C, and 3 washes with post-hyb wash 3 (50% formamide, 0.15M NaCl, 1X PE, 0.1%

Tween-20) at 50°C. Hybridized probes were detected by immunohistochemistry. Samples were blocked in 2mM levamisole and 10% goat serum in TBST for 1 hour with gentle rocking. Samples were then incubated with anti-DIG-alkaline phosphatase Ab (Boehringer Mannheim) diluted 1:5000 in blocking solution overnight at 4°C, washed 6 times with TBST containing 0.5 mg/mL levamisole, and finally washed 3 times in freshly prepared NTMT (0.1M NaCl, 0.1M Tris pH 9.5, 0.2M $MgCl_2$, 0.01% Tween-20). Colour reactions were performed by incubation of samples with 4.5 μL/mL NBT and 3.5 μL/mL BCIP (NBT/BCIP Combo, GibcoBRL) in NTMT for 5 to 20 minutes to yield a blue colour. Samples were counterstained with hematoxylin for 5 minutes and photographed by digital image capture software (CoolSnap for Mac) under a dissecting microscope.

Gelatin-substrate Zymography

Conditioned media collected from villous explant cultures were examined for changes in release of gelatin-degrading proteinases by gelatin-substrate zymography. Aliquots of conditioned medium were harvested at various days of culture and 7 μL of media was solubilized in non-reducing buffer and electrophoresed in 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, SDS was removed from the gel by 2.5% Triton X-100 washes (2 times 15 min). The gel was then washed in 1X developing solution (Novex) and incubated in the same buffer at 37°C overnight. The presence of gelatinase activity was detected by determining zones of lysis after Coomassie Brilliant Blue staining. Gelatinolytic activity was detected as clear bands against purple/blue-stained gelatin background.

3.3 Results

Carbenoxolone inhibits GJIC in JAr trophoblast cells and in placental villous outgrowth EVT cells

To determine whether or not CBX, which is known to inhibit GJIC in other cell types, can inhibit GJIC in trophoblast cells, two methods were used. First, cell coupling was assessed after treatment of a trophoblast choriocarcinoma cell line (JAr) with CBX (125 μM) for 24 hours by the scrape-loading technique (Figure 3.1, A). Although Lucifer-Yellow transferred through many layers of cells in both untreated and GZA control analog-treated cells (A, panels a and b),

CBX treatment resulted in the dye being maintained in cells adjacent to the scrape line (A, panel c). This demonstrates that CBX treatment can block GJIC in trophoblast cells that express predominantly Cx40. To determine whether or not CBX at 125 μM had any toxic effects on these trophoblast cells, JAr cells were treated with CBX (125 μM) or GZA (125 μM) for 24 hours and cell viability was assessed by fluorescein diacetate-propidium iodide staining methods. Treatment with CBX did not increase the number of dead cells (red cells, Figure 3.1 B, panel c) compared to untreated or GZA-treated controls (Figure 3.1 B, panels a and b), indicating that CBX is not directly cytotoxic to JAr trophoblast cells.

The ability of CBX to inhibit GJIC was also directly determined in placental villous explant cultures by micro-injection techniques. In control explants, the fluorescent dye Calcein-AM micro-injected into EVT cells within the outgrowth spread to a group of neighbouring cells by 200 sec (Figure 3.2, A). In contrast, treatment of explants for 1 hour or 24 hours with CBX resulted in the retention of Calcein-AM in the micro-injected cell for up to 250 sec. (Figure 3.2, B). Therefore, CBX treatment is also able to block GJIC in villous explant outgrowth EVT cells.

Inhibition of GJIC alters the phenotype of placental villous explants

To determine the effects of inhibiting GJIC on the phenotype of villous explant outgrowths, three inhibitors were used. The first, CBX, was found to inhibit GJIC in both JAr trophoblast cells and in placental villous explant outgrowth EVT cells. Heptanol was not specifically tested for its ability to inhibit GJIC, but was found to elicit identical effects on placental explant morphology and phenotype, suggesting that it too blocked GJIC. Oleamide, on the other hand, did not elicit any effect on explant outgrowth morphology, even at relatively high (100 μM) concentrations. Therefore, it was not used in subseqent phenotypic analyses. The similar effects of CBX and heptanol on explant morphology and phenotype suggest that the primary mechanism of action of these reagents is in the blockade of GJIC. Oleamide was shown to be ineffective in educing any morphologic effect on placental outgrowth cultures, and it is known to be only a partial uncoupler in other cell-types (219).

GJIC blockade results in a change in EVT outgrowth morphology

To determine the effect of GJIC blockade on villous explant outgrowth morphology, explants were cultured for 3 days on Matrigel at 3% O_2 to allow outgrowths to form from villous

Figure 3.1. Dye-transfer and cell viability of JAr cells following CBX treatment. (A) Scrape-loading assessment of GJIC in JAr cells. In untreated and GZA-treated control cells, Lucifer-yellow is transferred through multiple cell layers away from the scrape line (a and b). In CBX-treated cells, Lucifer-yellow is maintained in cells along the scrape line, indicating the loss of functional cell-cell coupling. CBX treatment does not affect cell viability, as determined by FDA-PI staining (B). Under green fluorescence all cells are detected (a, b, c), whereas under red fluorescence dead cells are revealed (*a, b, c*). There is no significant increase in cell death with CBX treatment (*c*), compared to untreated or GZA-treated controls (*a, b*).

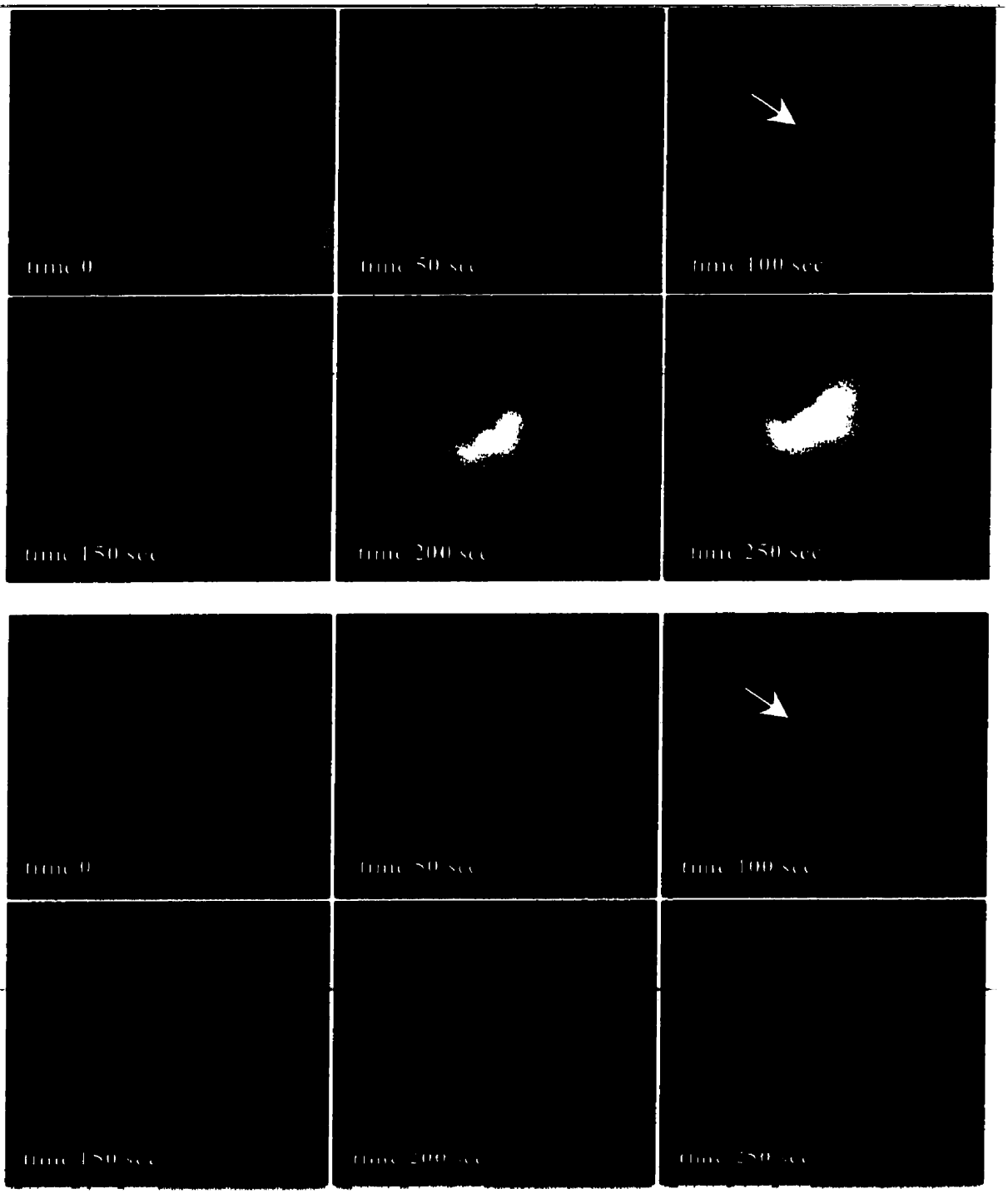

Figure 3.2. Time-lapse micrographs of micro-injected EVT cells within placental villous explant outgrowths. (A) A control explant EVT cell micro-injected with Calcein-AM demonstrates passage of the dye to neighbouring cells by 200 sec. (arrow indicates micro-injected cell). (B) Treatment of explants with CBX for 24 hrs or 1 hr results in the retention of Calcein-AM in the micro-injected cell for up to 250 sec. (arrow indicates micro-injected cell).

tips. Photos were taken at this time (just prior to treatment) and again 24 hours after treatment with either CBX (125 μM) or heptanol (5 mM). The outgrowths that form in culture from the tips of placental villi result from *de novo* proliferation of CTB cells proximal to the villous tip and the constant migration of these in column-like groups away from the tip. Outgrowths will usually emerge from all villous tips in one placental explant (between 3 and 6 tips), although sometimes one or two tips do not form outgrowths. The molecular basis for this observation is unknown. Furthermore, viability of placental explants cultured from one placenta is seldom 100% - on average approximately 20% of explants either do not attach to the Matrigel or do not form outgrowths. These explants are considered dead prior to treatment (as determined by a change in colour of the tissue to a pale brown) and are not included in the treatment groups. In untreated explants cultured at 3% O_2, the outgrowths that form are organized and compact, with streams of EVT cells migrating along the Matrigel (Figure 3.3, a-c). Exposure of placental explants to CBX or heptanol resulted in an obvious change in the morphologic structure of outgrowths (Figure 3.3, c-f). The outgrowths became much more diffuse, the organization of EVT cells into migrating streams was abolished, and most cells became detached from the group and moved individually into the surrounding Matrigel. In explants treated with GZA, the control analog of CBX that does not inhibit GJIC, there was no difference in explant outgrowth morphology compared to media alone explants (Figure 3.3, a).

The differences in outgrowth morphology were very apparent when explants were sectioned at 6 μM for histologic examination. Sections were immunostained with HLA-G, which specifically stains outgrowth EVT cells in explants, in order to clearly identify the EVT cells. Again, in untreated and GZA-treated control explants, outgrowths emanating from the tips of villi are compact and organized, with streams of cells migrating into the Matrigel (Figure 3.4, A and B). Treatment with CBX results in the scattering of EVT cells, leaving clear spaces between cells or groups of cells. The outgrowths also lost their organization and there were no streams of cells (Figure 3.4, C). The change in outgrowth morphology observed following treatment with GJIC inhibitors was reminiscent of a more invasive phenotype, but in order to evaluate this further, molecular markers of EVT cell differentiation along the invasive pathway were assessed.

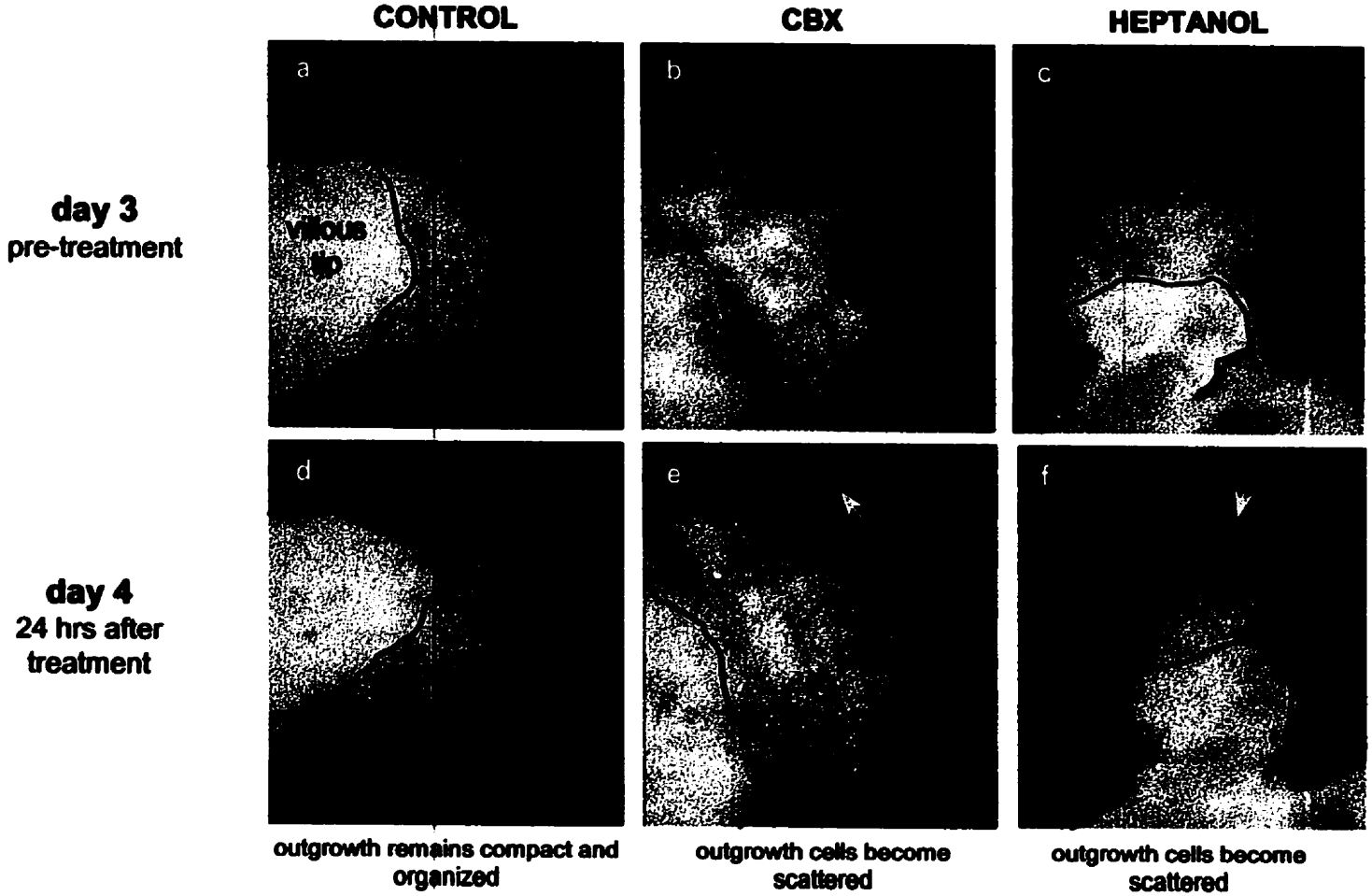

Figure 3.3. The effect of GJIC-inhibitors on placental villous explant outgrowth morphology. Placental villous explants (from 6-8 week placentas) were cultured in 3% O_2 on Matrigel and allowed to grow *de novo* EVT outgrowths over 3 days (blue lines delineate the original edge of the villous at the time of culture, black arrows show typical streams of EVT cells). Explants were then treated with GZA control analog (a and d) or the GJIC-inhibitors CBX (e) and heptanol (f). Note the dissociation and loss of organization of outgrowth EVT cells upon CBX and heptanol treatments (e and f), but not with GZA control treatment (d) (light blue arrows point to individual cells detached from the outgrowth). (magnification=60X)

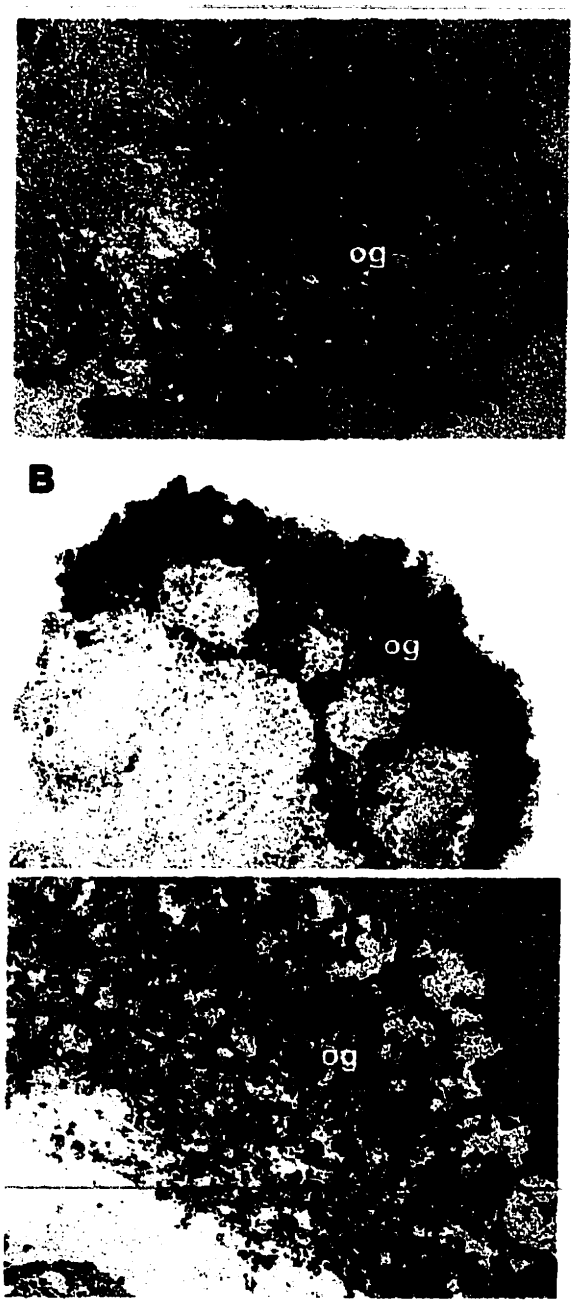

Figure 3.4. Histologic morphology of EVT outgrowths in untreated, GZA-treated control, and CBX-treated explants. Explants were paraffin-embedded and sectioned at 6 μM. All panels show sections immunostained with anti-HLA-G to identify EVT cell outgrowths (brown or black staining). (A and B) In untreated (A) and GZA-treated (B) control explants, outgrowths are compact and organized, with streams of cells migrating into the Matrigel. (C) Treatment with CBX for 24 hrs results in the scattering of EVT cells, and outgrowths appear less compact and disorganized. (og=EVT outgrowth, magnification=200X)

Characterization of trophoblast differentiation in villous explant cultures treated with GJIC inhibitors

To characterize the phenotype that results from treatment of placental explant cultures with GJIC inhibitors, various molecular markers of trophoblast differentiation were evaluated. The first was the proliferation antigen marker Ki67, which is usually expressed in villous stem CTB cells and in EVT cells in outgrowths. Since treatment with GJIC inhibitors resulted in a morphological change that was suggestive of a more invasive phenotype, we hypothesized that proliferation would be decreased. Immunohistochemical staining with a monoclonal antibody to Ki67 detected proliferating cells (as indicated by darkly stained nuclei) in EVT cells in outgrowths of GZA-treated control placental explants after 4 days in culture, especially most proximal to the villous tip (Figure 3.5, B). In contrast, treatment with the GJIC inhibitor CBX (125 μM, 24 hrs) resulted in the abolishment of EVT cell proliferation within columns, although proliferating nuclei were detectable in villous stem CTBs underlying the outer STB layer (Figure 3.5, D). Therefore, CBX did not result in a global inhibition of proliferation, but rather an EVT cell specific abolishment of proliferation.

Matrix-metalloproteinases (MMPs) are known to facilitate the migration of EVT cell columns and invasion of individual EVT cells into the maternal decidua. In explant cultures of first trimester placental villi, it has previously been shown that active MMP-2 is released into the media (87). The activity of gelatin-degrading matrix-metalloproteinases in the present study was assessed by gelatin-substrate zymography. In control and GZA-treated explants, both pro-MMP-2 and MMP-2 were active through all days of culture. In contrast, treatment with CBX to block GJIC resulted in a decrease in MMP-2 activity in a time-dependent manner (Figure 3.6).

Trophoblast differentiation *in situ* is accompanied by a regulated switch in integrin repertoire. Immunohistochemistry experiments detected staining for α5 integrin in STB and outgrowth EVT cells in all explants; control, GZA-treated and CBX-treated (Figure 3.7). This integrin is characteristic of EVT cells in the cell columns. When EVT cells differentiate to a more invasive phenotype, α1 integrin expression is induced. However, there was no obvious induction of α1 integrin expression in placental explants treated with CBX, indicating that they are not triggered to differentiate to a late invasive phenotype (Figure 3.8). Staining for α1 integrin was observed in a positive control tissue (human fetal kidney) demonstrating that staining conditions were suitable to detect the protein.

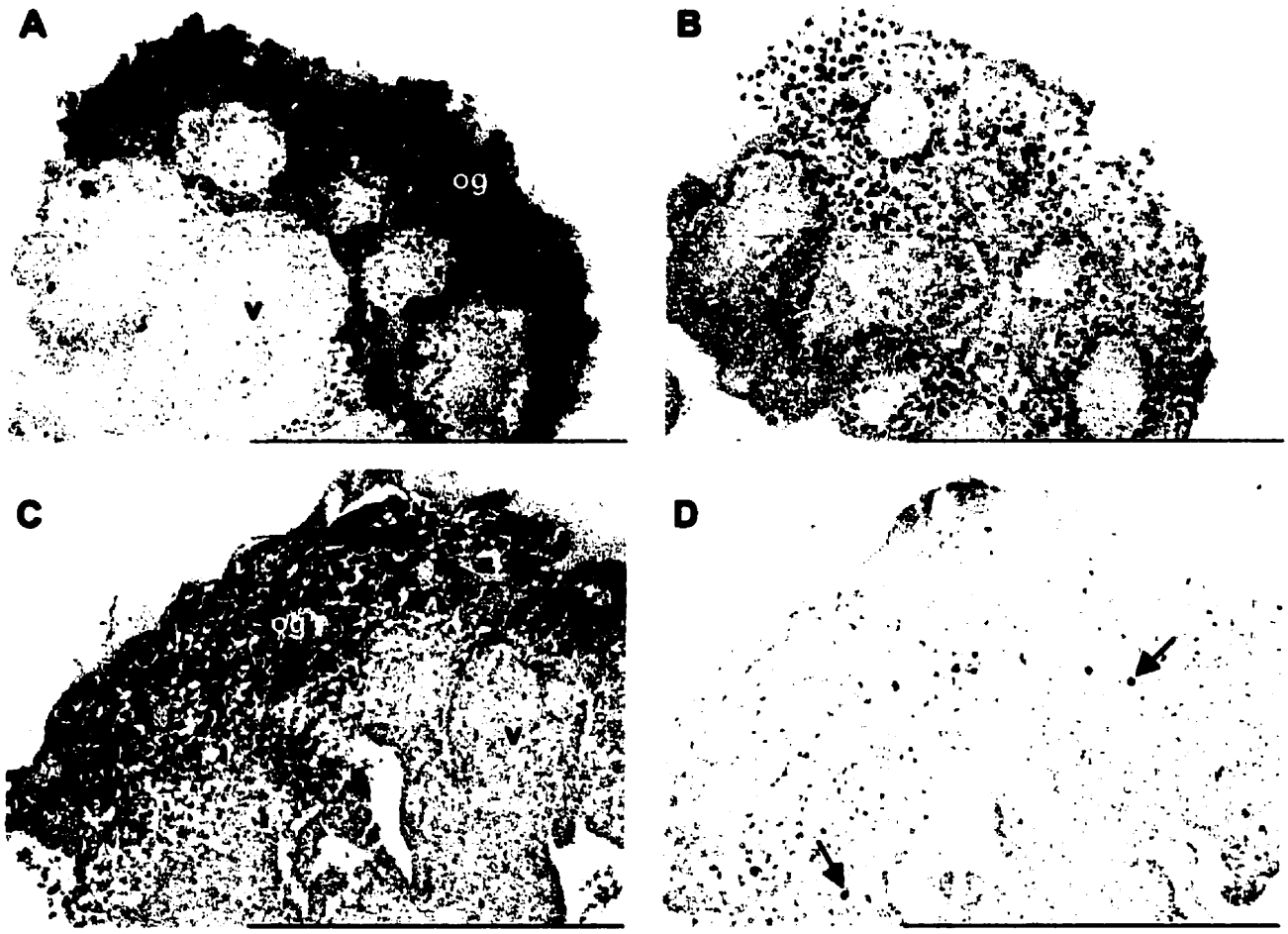

Figure 3.5. Immunohistochemical staining for the proliferation marker Ki67 in GZA-treated control and CBX-treated explants. (A and C) Black or brown staining identifies EVT cells in explant outgrowth, as determined by HLA-G immunostaining. (B) In GZA-treated control explants, many outgrowth EVT cell nuclei stain positive (black) for Ki67, indicating that they are proliferating. (D) CBX-treatment results in a complete lack of Ki67-positive nuclei in EVT outgrowths, although some proliferating villous CTB cells are present (arrows). (og=EVT outgrowth, v=villous, magnification= 200X)

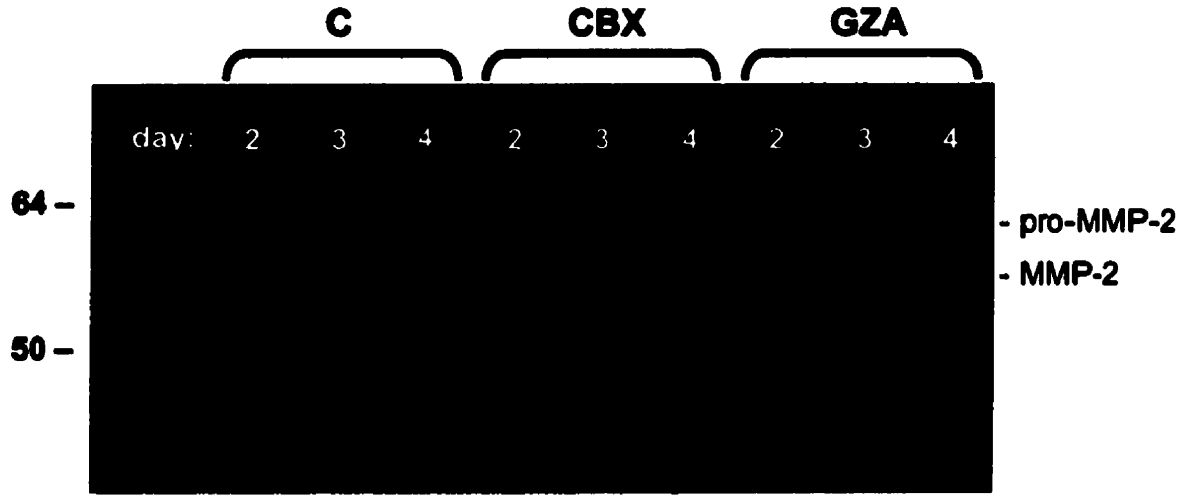

Figure 3.6. Gelatin-substrate zymography of conditioned media collected from untreated control, GZA-treated control, and CBX-treated placental villous explant cultures. In control (C) and GZA-treated control explants, pro-MMP-2 and MMP-2 activities are present from day 2 through day 4. Treatment of explants with CBX, on the other hand, results in a time-dependent decrease in both pro-MMP-2 and MMP-2 activities.

Figure 3.7. Immunohistochemical staining for alpha5 integrin in untreated, GZA-treated control, and CBX-treated explants. Paraffin-embedded explants were sectioned at 6 μM and immunostained for α5 integrin. In all groups, α5 integrin is detected in the STB (arrow in C), as well as in EVT cells of outgrowths. (A=untreated control, B=GZA-treated control, C=CBX-treated control) Outgrowths are bracketed. (og=EVT outgrowth, v=villous, magnification=200X)

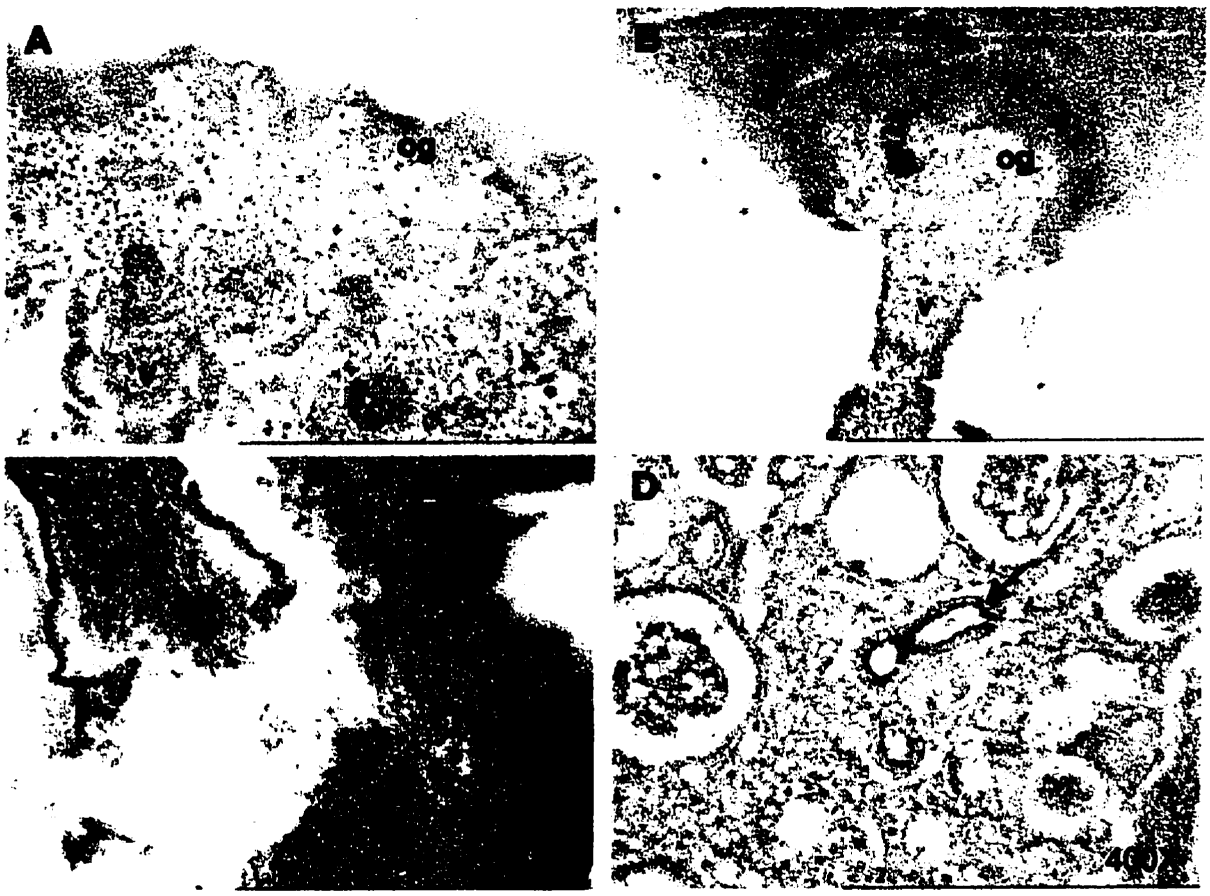

Figure 3.8. Immunohistochemical staining for alpha1 integrin in untreated, GZA-treated control, and CBX-treated explants. Paraffin-embedded explants were sectioned at 6 μM and immunostained for α1 integrin. In all groups, α1 integrin weakly detected or absent from EVT outgrowth. (A=untreated control, B=GZA-treated control, C=CBX-treated). No difference between groups was observed. (D) In a human fetal kidney control tissue section, positive α1 integrin staining is detected in tubule epithelial cells. (og=EVT outgrowth, v=villous, magnification=200X)

Cx40 mRNA is expressed in EVT cells in villous explant cultures

Because of the abundance of Cx40 in anchoring villous cell columns *in vivo*, we sought to determine whether Cx40 expression is maintained in villous explant outgrowths *in vitro*. To this end, *in situ* hybridization was performed to localize Cx40 mRNA. By both whole-mount and standard ISH protocols, Cx40 was detected in outgrowth EVT cells in control explants (Figure 3.9, A and B). By whole-mount immunohistochemistry and immunofluorescence, Cx40 protein was similarly detected in EVT outgrowths of control explants (Figure 3.9, C and D). This provides the first evidence of Cx40 expression in EVT cells in placental villous explants.

Treatment with anti-sense oligonucleotides to Cx40 alters the morphology of placental villous explants

In experiments identical to those performed for CBX and heptanol, placental villous explants were treated with phosphorothioated oligonucleotides synthesized towards sequences flanking the translation start site of Cx40. Explants were precultured for 3 days to allow the formation of an EVT cell outgrowth. Media containing anti-sense or sense oligonucleotides were then added to explants for 48 hours. After 24 hours of Cx40 anti-sense treatment, outgrowth EVT cells started to become rounded and more diffuse, similar to the effect observed of the non-specific GJIC inhibitors. The effect was not as pronounced as CBX or heptanol after 24 hours. When Cx40 anti-sense was left for 48 hours, the outgrowths more closely resembled those treated with CBX or heptanol for 24 hours (Figure 3.10). The outgrowths were very diffuse and lacked organization.

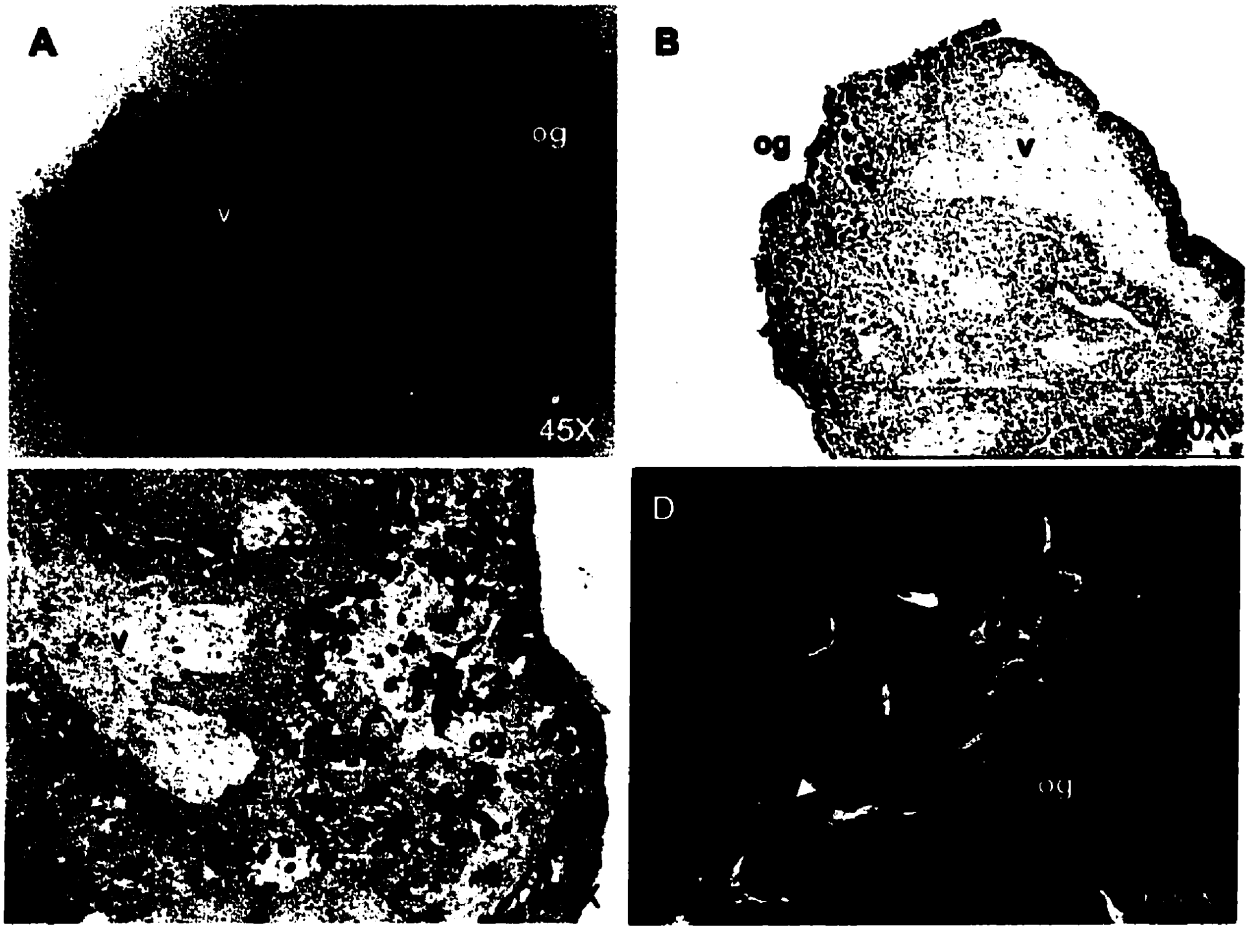

Figure 3.9. Cx40 mRNA and protein localization in placental villous explants. Cx40 mRNA was localized by whole-mount *in situ* hybridization (A) and standard *in situ* hybridization (B). Positive staining for Cx40 (dark blue against pink counterstain in A and B) was identified in all outgrowth EVT cells in control explants. Cx40 protein was also localized to EVT outgrowths by whole-mount immunohistochemistry (C) or standard immunofluorescence (D). (C) Dark blue staining indicates positive staining for Cx40 in outgrowth EVT cells in a control explant. Cx40 is not detected in villous stroma or villous CTB or STB (counterstained pink). (D) Punctate staining for Cx40 in an EVT outgrowth of a control explant (arrow). (og=EVT outgrowth, v=villous)

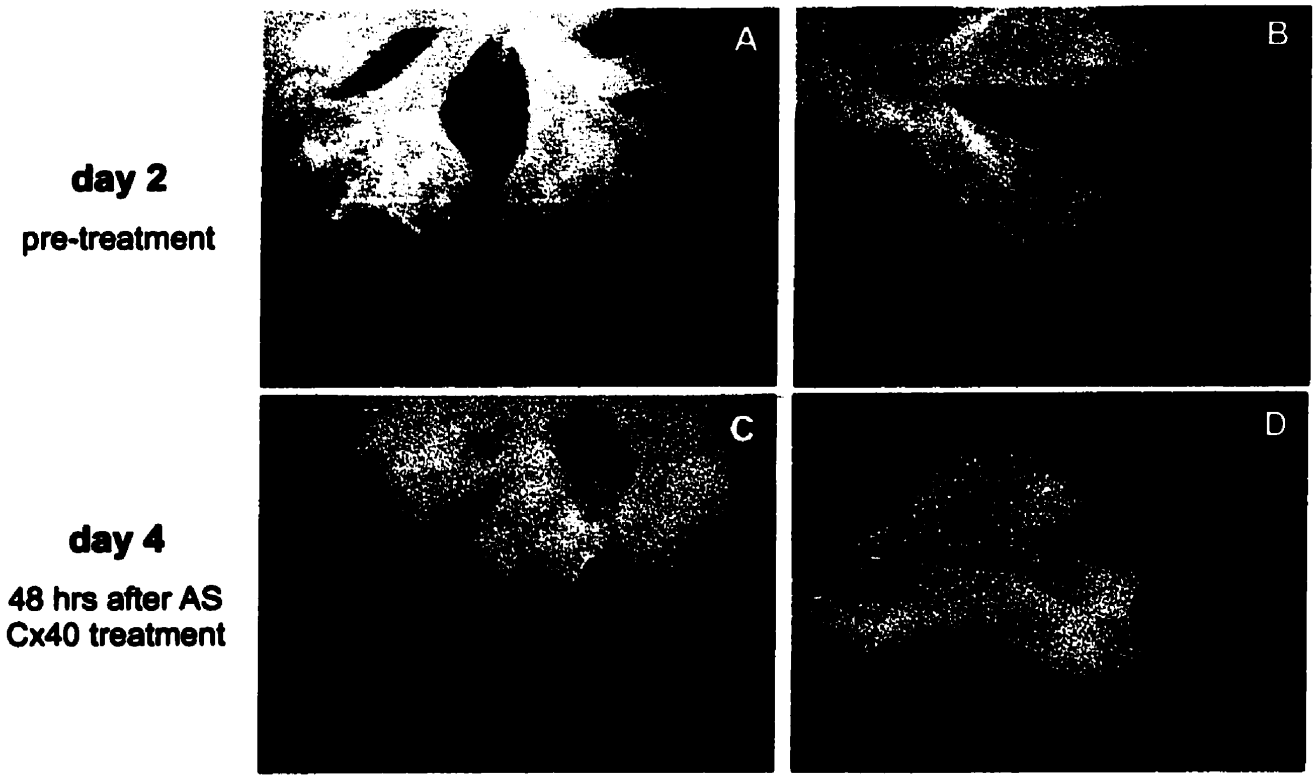

Figure 3.10. Treatment of placental explants with anti-sense oligos to Cx40 results in an identical change in outgrowth morphology as the general GJIC-inhibitors CBX and heptanol. Placental villous explants (from 6-8 week placentas) were cultured in 3% O_2 on Matrigel and allowed to grow *de novo* EVT outgrowths over 2 days. Explants were then treated with anti-sense oligos to Cx40 or scrambled control oligos. Note the profound movement of outgrowth EVT cells away from the villous tip and the complete loss of organization upon anti-sense treatment (C), compared to scrambled control treatment (D). (magnification=40X)

3.4 Discussion

The data presented in this chapter provide evidence that intercellular communication via gap junctions in EVT cell columns maintains the cells in a proliferative and organized column. Gap junction proteins are expressed in proliferating EVT cells in anchoring cell columns *in vivo*, and expression is maintained in EVT outgrowths emanating from placental villous explant cultures *in vitro*. In this study, Cx40 mRNA and protein were detected in outgrowth EVT cells when 7-week placental explants were cultured in 3% O_2 for up to 6 days. To assess the role of GJIC in EVT outgrowths, placental explants were cultured for 3 days to allow outgrowth formation and treated with two gap junctional communication inhibitors, CBX and heptanol for 24 hrs. Carbenoxolone is also known to inhibit 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2) activity, an enzyme present in STB that converts cortisol to cortisone. We do not believe that this effect of CBX would have any impact on the results obtained. Firstly, there is no evidence that placental villous explants produce cortisol, and they were cultured in serum-free conditions, so no exogenous cortisol was present. Secondly, GZA also inhibits 11β-HSD-2, and treatment with GZA did not affect explant morphology or molecular phenotype. The ability of CBX to block GJIC in trophoblast cells was assessed by the scrape-loading technique in JAr cells (a trophoblast cell line that expresses predominantly Cx40) and by micro-injection of individual EVT cells in outgrowths of villous explant cultures. In both cases, CBX inhibited functional coupling between trophoblast cells. Treatment of placental villous explants with CBX or heptanol resulted in a dramatic change in outgrowth morphology, in that cells that were once compact and organized in streams became diffuse and disorganized. Individual cells were always detected in outgrowths following GJIC-blockade, reminiscent of a more invasive phenotype. To assess the phenotype following blockade of GJIC, four markers of trophoblast differentiation along the invasive pathway were analyzed. By immunohistochemistry, we determined that Ki67 expression was abolished in outgrowth EVT cells, indicating that GJIC-blockade results in the inhibition of EVT cell proliferation. Matrix-metalloproteinase activity was examined by gelatin-zymography, and MMP-2 activity was found to decrease with treatment with either CBX or heptanol. MMP-2 is thought to play dual roles in anchoring cell column biology. First, it is thought to be required in column formation to allow villous CTB cells to leave the basal lamina and migrate out of the villous. Therefore, proliferative EVT cells

most proximal to the villous produce MMP-2. MMP-2 is then thought to be down-regulated in the distal column and early invasive phenotype. EVT cells deep in the maternal decidua re-express MMP-2 to facilitate invasion. The decrease in MMP-2 activity determined in this study suggests that GJIC is required to maintain outgrowth EVT in the early proliferative phenotype. The expression of α5 and α1 integrins did not change upon GJIC-inhibition, demonstrating that the EVT cells are not triggered to differentiate towards the highly invasive phenotype characterized by α1 integrin. Together, these results reveal the requirement of GJIC in maintaining proliferative EVT outgrowths, and suggest that a down-regulation of GJIC may allow for an exit from the proliferative phenotype.

The results of this study are in accord with the proposed role of GJIC to coordinate groups of cells during development processes. For example, neural crest cells that migrate from the neural tube to the outflow tract of the heart require GJIC through channels composed of Cx43 (171). These cardiac crest cells endogenously express Cx43, and in Cx43-deficient mice, cardiac crest cells do not properly migrate to the outflow tract. It has been suggested that gap junctions along streams of migrating crest cells provide a pathway for signaling molecules to pass from cell to cell, thereby equilibrating their intracellular environments to synchronize gene expression, kinase activity etc. In the case of EVT cell column migration, it is possible that decidual-derived chemotactic molecules bind to receptors on EVT cells at the leading edge of the cell column, which would result in a change in intracellular signaling molecules such as cAMP, cGMP, or Ca^{2+} in these cells. The presence of functional GJIC could then transduce this signal to adjacent cells along the migrating column via passage of cAMP, cGMP, or Ca^{2+}. The coordination of cells along a stream or branch is evident in the conduction system of the heart, which requires gap junctions to pass electrical impulses from the AV node through the ventricular conduction system to coordinate myocardial contractions (181).

Placental explant outgrowths in low oxygen (3% O_2) are known to be maintained in the early, proliferative EVT cell phenotype (241). This low oxygen environment is similar to the *in vivo* situation in the early placenta (prior to 9-10 weeks), when maternal vessels are not yet breached, limiting blood flow to the placenta. During this early period, EVT invasion must be occurring in order for maternal vessels to be breached. Genbachev *et al.* propose that the gradient of oxygen tension along an anchoring EVT cell column may influence differentiation of EVT cells towards the invasive phenotype (135). Interestingly, GJIC is influenced by changes in

oxygen tension in cultured human umbilical vein endothelial cells (HUVECs) and astrocytes. Zhang *et al.* have shown that when HUVECs are cultured in low oxygen conditions (0.1% O_2) and then transferred to normal atmospheric conditions (20% O_2) for 2 hrs, there is a 20% reduction in GJIC between the cells (242). When tyrosine kinase activity was inhibited by treatment with genistein, the reduction in GJIC upon reoxygenation was abolished. They conclude based on this and other data that upon reoxygenation, Cx43 becomes tyrosine phosphorylated, which decreases GJIC. In astrocytes cultured in low oxygen (1% O_2), a change in the phosphorylation level of Cx43 is not involved in the decrease in GJIC observed upon reoxygenation. Instead, Martinez and Saez have determined that arachidonic acid metabolites such as prostaglandins or thromboxanes are required for the decrease in GJIC observed in astrocytes following 90 min of reoxygenation (243). In these studies, astrocytes were cultured in 1% O_2 (which did not affect cell-cell coupling) and then reoxygenated for 15 to 300 minutes in the presence or absence of indomethacin, a prostaglandin H synthase inhibitor. Reoxygenation resulted in a maximal 65% reduction in GJIC at 90 minutes, which recovered by 300 min post-reoxygenation. The reduction in GJIC was prevented by pre-treatment with indomethacin, which inhibits the metabolism of arachidonic acid to PGH_2, the substrate for specific prostaglandin and thromboxane synthase enzymes.

Arachidonic acid metabolites are present at the fetal-maternal interface. As the EVT column migrates deeper into the decidua and encounters a higher oxygen environment ('reoxygenation'), tyrosine kinases or arachidonic acid metabolites could cause the transient inhibition of GJIC, which would inhibit proliferation and allow for individual EVT cells to detach from the column (according to the data presented in this chapter).

If the inhibition of GJIC is a requirement for EVT differentiation *in vivo*, there are many bioactive molecules and growth hormones that may act on EVT cells to inhibit GJIC in distal cell columns. Nitric oxide (NO) has been shown to inhibit GJIC in cultured astrocytes (244). Ahmed *et al.* have demonstrated that treatment of cultured first trimester trophoblast cells with VEGF (10 ng/mL) causes a 60% increase in NO release through activation of the constitutive isoform of NOS (245). Since decidual macrophages are the principal source of VEGF at the materno-fetal interface, EVT cells at the leading edge of migrating cell columns may bind decidual-derived VEGF via Flt-1 receptors, which they are known to express, thus increasing NO release, which could then inhibit GJIC. Furthermore, VEGF can directly disrupt GJIC in

endothelial cells via signaling cascades involving both c-Src and mitogen-activated protein kinases (246). The VEGF-related peptide PlGF is known to inhibit basal NO release from cultured first trimester trophoblast cells (247). Interestingly, in IUGR pregnancies PlGF is abnormally high. IUGR is characterized by an increase in CTB proliferation and a poorly perfused placenta (247). The high PlGF in IUGR could cause a decrease in NO production, which could then lead to abnormal maintenance of GJIC in trophoblast cells, sustaining these cells in a proliferative, undifferentiated phenotype incapable of invasion (resulting also in poor placental perfusion).

As discussed in Chapter 1, HGF has been implicated in promoting trophoblast invasion. Cartwright *et al.* propose that this action of HGF is mediated through NO production, since the inhibition of NO production abolishes HGF-induced trophoblast invasion (77). It is possible that HGF-induced NO production could inhibit GJIC, which would favor trophoblast invasion. However, whether NO can inhibit GJIC in trophoblast cells is not currently known and deserves further study.

In cells of epithelial origin, EGF and PDGF have been reported to block GJIC. Both of these growth factors have been studied for their putative role in mediating EVT proliferation and/or invasion. EGF is thought to stimulate EVT cell invasion – the addition of EGF to first trimester CTB cultures produces a several-fold increase in invasive capacity (38). The authors of this study suggest that maternal EGF may be acting on EVT cells *in vivo* to promote trophoblast invasion. Perhaps binding of decidual-derived EGF to its receptor on EVT cells at the distal edge of anchoring columns inhibits GJIC, which then allows for differentiation towards the invasive phenotype. PDGF has been studied for effects on isolated term CTBs, but not on isolated first trimester CTBs. Villous CTBs do not express PDGF receptors, but invasive EVT have been reported to express both PDGF receptors (100). These receptors may support PDGF-induced inhibition of GJIC. However, the effects of PDGF on trophoblast GJIC have yet to be defined.

TGF-β, a growth factor known to inhibit trophoblast invasion, has been shown to increase Cx43 synthesis, content, and apparent half-life in bovine aortic endothelial cells (BAEC) (248). If TGF-β also upregulates Cx40 in anchoring EVT cell columns, it could inhibit invasion by sustaining GJIC, thus maintaining the cells in a proliferative and undifferentiated state. The role of TGF-β3 has been examined in placental villous explant cultures grown at 3%

O_2, where Caniggia *et al.* found that inhibition of TGF-β3 expression by anti-sense oligonucleotides resulted in a change in outgrowth EVT phenotype from proliferative to invasive (134). If TGF-β3 acts to maintain Cx40 expression and GJIC, a decrease in TGF-β3 could decrease Cx40 expression, resulting in diminished GJIC and hence differentiation towards an invasive phenotype. However, in BAEC cells TGF-β1 also prevented the normal increase in Cx37 expression (248). Therefore, the effect of TGF-β on Cx40 in trophoblast cells should be evaluated.

IGF is another growth factor that has been shown to enhance EVT cell invasion and inhibit GJIC. Homma *et al.* have conducted experiments with Cx43 channels reconstituted into oocytes and found a time-dependent decrease in coupling with IGF treatment (249). The authors determined that the C-terminal cytoplasmic domain of Cx43 is required for this uncoupling effect, and conclude that a 'ball and chain' gating mechanism closes Cx43 gap junctions upon IGF and insulin receptor activation. They propose that phosphorylation of serine 279 may be involved in this IGF-induced uncoupling process. In the developing human placenta, IGF-II mRNA is expressed throughout gestation only by EVT cells. This localization supports a role for IGF-mediated GJIC-inhibition in EVT differentiation and invasion.

Another intriguing aspect of gap junctions is that they are associated with cadherins. Different cadherins are thought to recruit different connexins to the cell membrane in order to form gap junctional plaques. Fujimoto *et al.* have studied gap junctions and cadherins in hepatocytes, and conclude that E-cadherin expression provides a foci for gap junction formation (250). Furthermore, transfection of rat epithelial cells and rat fibroblasts with E-cadherin has been shown to increase GJIC communication between these two cell-types by 10-fold (115). In anchoring EVT cell columns in the human placenta, E-cadherin is expressed in the proliferative column. The presence of E-cadherin may promote the formation of gap junctions composed of Cx40 in cell columns. The presence of GJIC would then maintain the cells in the proliferative phenotype, based on the results in the present study. E-cadherin is down-regulated in the distal portion of cell columns, where EVT cells begin to differentiate towards the invasive phenotype. The lack of E-cadherin could result in the loss of a recruiting mechanism for connexins to the cell membrane, and thus decreased GJIC. In experiments using antibodies to functionally block cadherins in freshly isolated CTB cells seeded on Matrigel-coated inserts, Zhou *et al.* have found

that blocking E-cadherin results in a 3-fold increase in trophoblast invasiveness. It is possible that this effect is mediated by a decrease in GJIC resulting from E-cadherin blockade.

E-cadherin has been examined with respect to changes in connexin expression during murine trophoblast differentiation and invasion. In the early murine placenta (before 6 days pc), trophoblasts express E-cadherin, but no connexins are detectable. With invasion of the polar trophoblast into the maternal decidua from day 7 pc onwards, E-cadherin expression is lost and Cx31 mRNA and protein is induced (251). Thus, during murine placental development, E-cadherin is expressed in the proliferative phenotype and expression is lost in the invasive phenotype, similar to the human situation. In the mouse, however, proliferative trophoblasts do not express connexins, whereas proliferative human EVT express both Cx40 and Cx45. When murine trophoblasts differentiate into giant cells, Cx31 and Cx43 are induced, in contrast to invasive EVT cells in the human, which lose connexin expression. The explanation for these data may lie in the pronounced differences in structure and function of the placenta between the two species.

The precise mechanisms by which GJIC-inhibition may cause a change in EVT cell phenotype are currently unknown. Similar phenomena have been reported in other cell types, such as osteoblasts and myoblasts. When rat osteoblastic cells are treated with anti-sense cDNA against Cx43, there is a decrease in alkaline phosphatase activity and osteocalcin levels, two phenotypic markers of mature osteoblastic cells (175). Inhibition of GJIC in myoblasts by CBX-treatment results in the prevention of the normal *in vitro* differentiation program of myoblasts to syncytialize into myotubes (176). Normally, cultured myoblasts are induced to differentiate into myotubes by serum withdrawal, in a process that requires the induction of the bHLH transcription factors myogenin and MRF4. When myoblasts are treated with CBX and then stimulated to differentiate by serum withdrawal, the activation of the bHLH factors required for differentiation is inhibited, and the cells are blocked in a non-proliferative, pre-differentiated state. In the developing human placenta, bHLH transcription factors are thought to regulate the differentiation of EVT cells from the proliferative to invasive phenotype in anchoring cell columns. In purified CTB cell cultures, differentiation towards the invasive phenotype corresponds with a decrease of the bHLH transcription factors Mash2, Id-2 and E-factor (28). Since bHLH induction is inhibited by GJIC-blockade in myoblasts, it is tempting to speculate that GJIC-blockade in villous explant cultures inhibits Mash2, Id-2 or E-factor function or

expression, resulting in a change in outgrowth EVT phenotype to a non-proliferative, pre-differentiated state, as assessed in the present study. This could be tested by evaluating the expression of Mash2, Id-2 and E-factor mRNA or protein following gap junctional blockade in human placental explants. Even if expression of these transcription factors is not altered, it is possible that they are instead functionally inhibited, for example by phosphorylation or cytoplasmic localization.

The results of these studies demonstrate a novel mechanism regulating trophoblast differentiation and invasion. Gap junctions are required for proliferative EVT cell columns, since inhibiting GJIC with potent uncoupling reagents (CBX and heptanol) resulted a change in morphology and phenotype of EVT ougrowths emanating from villous tips in explant culture. The data obtained in these experiments, together with the wealth of information on the effects of growth factors, cytokines, and oxygen tension on both trophoblast phenotype and GJIC, suggest a new model for trophoblast differentiation (Figure 3.11). Decidual-derived growth factors and cytokines may bind to receptors on EVT cells at the distal edge of migrating cell columns, inhibit GJIC through Cx40 channels, and thus inhibit proliferation to allow for differentiation towards an invasive phenotype. Additionally, the increase in oxygen tension encountered by a migrating column into the decidua could uncouple GJIC. Studies will be extended to determine Ki67 expression and MMP-2 activity following anti-sense Cx40 treatment, and protein levels will be determined to verify the silencing effect of the anti-sense. Anti-sense Cx40 treatment of explants resulted in a clear effect on morphology, but this effect was most apparent following 48 hours of treatment, whereas with the non-specific GJIC inhibitors the effect was striking by 24 hours. This difference in time-course of action may be in part due to the presence of Cx45 in EVT cell columns in the early placenta, as discussed in Chapter 2. The contribution of Cx45 to EVT cell column function could be assessed by treating explants with both Cx40 and Cx45 anti-sense oligos.

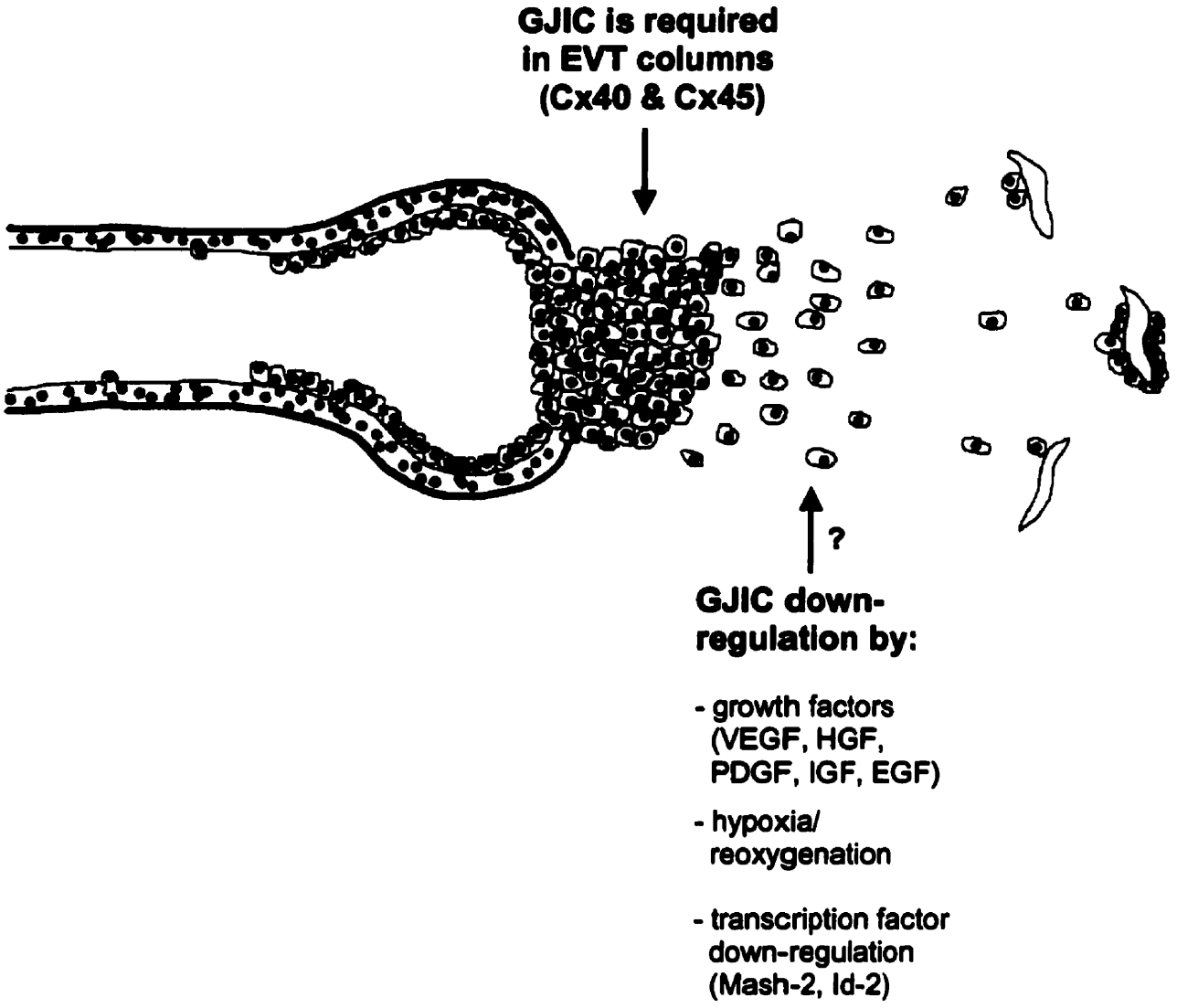

Figure 3.11. Model for the role of gap junctional communication in EVT differentiation and invasion. EVT cells in anchoring cell columns express Cx40 and Cx45, which may be required to co-ordinate the migration of columns into the maternal decidua and to maintain proliferation of column EVT cells. At the distal tip of the column, Cx40 expression and GJIC are down-regulated to allow individual EVT cells to detach from the column and differentiate into an invasive phenotype.

CHAPTER 4

OVERALL SUMMARY

The complex process by which EVT cells differentiate from the proliferative phenotype in anchoring cell columns to the invasive phenotype is only beginning to be understood at the molecular level. Anchoring column EVT cells are crucial for proper placentation because of their required role to invade the maternal decidua and remodel decidual and myometrial arteries. In the absence of this process, maternal vessels remain constricted and there is inadequate blood flow to the intervillous space bathing placental floating villi. Ultimately, this results in a poorly perfused hypoxic placenta, and an undernourished developing fetus, such as in IUGR pregnancies. Preeclampsia is characterized by the inability of EVT cells to remodel spiral arteries. Current models propose that in preeclampsia, EVT cells fail to adopt the 'vascular' phenotype characterized by $\alpha 1$ integrin and VE-cadherin, thus inhibiting their function as 'vascular' cells around maternal vessels.

In the present study, gap junctions were examined for their potential role in EVT differentiation in the early human placenta. Gap junctions are expressed in most tissues and are known to be developmentally regulated in many tissues, including the rodent placenta (251). In the first trimester human placenta, five connexin transcripts were identified by RT-PCR; Cx32, Cx37, Cx40, Cx43 and Cx45. The expressions of all of these, with the exception of Cx43, were temporally regulated throughout the first trimester. Localization of connexin protein expression by immunostaining provided information to speculate on their potential roles during placentation. Cx32 was found at the interface between villous CTB and STB layers in 6 week placentas, indicating that it may be important in mediating the early fusion of CTB cells to STB. Since Cx32 is expressed during the fusion of human EVT cells to giant cells in the decidua, this role in syncytial fusion seems plausible. Cx40 and Cx45 were both localized to anchoring column EVT cells. In particular, Cx40 expression in this population was intense and retained throughout the first trimester. Cx45 expression, on the other hand, was weak and was down-regulated in EVT cell columns at 9-10 weeks gestation, when oxygen tension in the placenta is known to increase. Cx45 may be regulated by oxygen tension, and could play an accessory role to Cx40 in anchoring cell columns when oxygen tension is low. Villous mesenchymal cells also demonstrated positive staining for Cx45, with intensity increasing with gestational age. Cx43 expression was also prominent in the mesenchyme. Since glucose and other maternal factors

must be transferred through the villous mesenchyme to reach fetal villous vessels in the early placenta, gap junctions may provide a rapid pathway for the transfer of these small molecules.

In terms of EVT differentiation, the specific localization of Cx40 to anchoring cell columns implicated gap junctions in anchoring column function. Coincident with these studies, another group was able to show that Cx40 expression decreases in individual EVT cells that detach from the cell column to invade the decidua (213). This suggested that a down-regulation of gap junctions was important in trophoblast differentiation towards the invasive phenotype. To address this hypothesis, placental villous explant cultures were treated with known GJIC-inhibitors and changes in EVT phenotype were assessed. When placental villous explants from 6-8 week gestation placentas are cultured on Matrigel in low oxygen conditions (3% O_2), outgrowths of EVT cells analogous to proliferative EVT cell columns *in* vivo form. Treatment of explants with the GJIC-inhibitors CBX and heptanol resulted in a change in the morphology and phenotype of these outgrowths. Instead of being compact and organized with streams of EVT cells migrating away from villous tips, outgrowths following GJIC-blockade became scattered and disorganized, with cells detaching from their neighbours. Analyzing the phenotypic alterations following GJIC-inhibition revealed the abolishment of EVT proliferation and MMP-2 production, two markers of the proliferative phenotype. Invasive markers such as α1 integrin and MMP-9 were not induced by GJIC-blockade. These results suggest that GJIC is required to maintain EVT outgrowths in the proliferative phenotype, and that blockade of GJIC results in EVT arrest in a post-mitotic, pre-invasive state.

Although the two GJIC-inhibitors used in this study are widely used as reagents to block GJIC in *in vitro* experiments, to specifically target gap junctions composed of Cx40, an anti-sense oligonucleotide strategy was used. A short (18-mer) phosphorothioated oligonucleotide was designed against the sequence flanking the mRNA translation start site. Such an approach is effective in inhibiting protein expression for other genes in placental villous explant cultures. Treatment of first trimester villous explants with anti-sense to Cx40 resulted in a change in outgrowth morphology similar to that observed with the non-specific GJIC-inhibitors CBX and heptanol. The effect was not as prominent after 24 hrs, but by 48 hrs the outgrowths were very scattered and disorganized, and the EVT cells of the outgrowth were rounded. This observation substantiates our hypothesis that gap junctions are required to maintain proliferative cell columns, and demonstrates the requirement for gap junctions composed of Cx40 in particular.

Future Directions

The results presented in this thesis describe a role for GJIC in maintaining proliferative cell columns during early human placental development. The effect of Cx40 anti-sense oligonucleotides on the morphology of first trimester villous explant outgrowths was determined, but the resulting phenotype has not been assessed. To more fully characterize the effect of Cx40 anti-sense oligo treatment, explants should be embedded, sectionned, and immunostained for Ki67 to assess proliferation, and α5 and α1 integrins. Conditionned media collected from treated explants should also be subjected to gelatin-zymography to determine changes in MMP activity. To fully analyze the change in phenotype following CBX or anti-sense Cx40 treatment of placental villous explants, DNA microarray technology could also be used. In such experiments, RNA would be extracted from control explants cultured at 3% O_2 and CBX or anti-sense Cx40 treated explants, reverse-transcribed with different fluors to label each group, and assessed for differences in mRNA abundance on a human cDNA microarray chip. Current human microarray chips contain 19,000 or 40,000 genes.

There are many growth factors and bioactive molecules that are known to induce trophoblast invasion and inhibit GJIC in various cell types. The effects of these factors on stimulating trophoblast invasion may be partially due to the inhibition of GJIC. Decidual-derived or EVT-derived growth factors may act on EVT cells in the most distal portion of anchoring cell columns to inhibit GJIC, thereby permitting their escape from the proliferative column. Examples of growth factors that could act in this manner include VEGF, HGF, IGF, PDGF, and EGF. The major inhibitor of EVT differentiation, TGF-β, can induce connexin expression in cultured endothelial cells, and may also be able to induce Cx40 expression in EVT cell columns, although this remains to be determined. It would be of interest to determine whether the growth factors that stimulate trophoblast invasion are also able to inhibit GJIC in trophoblast cells. This could be done using isolated first trimester CTB cultured at 3% O_2 and the scrape-loading technique to assess cell-cell coupling.

Changes in oxygen tension may also be a mechanism by which GJIC is inhibited to allow EVT differentiation in the distal cell column. The current understanding of early EVT cell columns describes an oxygen gradient along a migrating column, since oxygen tension in the placenta is 18 mmHg compared to 40 mmHg in the decidua. In preliminary experiments not

included in this thesis, first trimester villous explants were cultured at 3% O_2 for 2 days and then transferred to 20% O_2 for 24 hrs, with or without treatment with CBX. Morphologic examination of these explants indicated that reoxygenation (transfer to 20% O_2) resulted in a subtle change in outgrowths reminiscent of the change that occurs with CBX-treatment of explants at 3% O_2. The outgrowth EVT cells became more rounded and began to dissociate from adjacent cells, resulting in a more diffuse appearance. In addition, proliferation of outgrowth EVT cells is abolished upon reoxygenation and MMP-2 activity decreases slightly, both of which also occur with GJIC-inhibition in explants cultured at 3% O_2. Concurrent treatment with CBX upon reoxygenation exacerbated the effects. These data suggest that reoxygenation of placental villous explants may inhibit GJIC, resulting in a change in EVT outgrowth morphology and phenotype. In order to verify this, villous explants transferred from 3% O_2 to higher a oxygen tension (around 12% O_2 would mimic the *in vivo* situation) could be assessed for GJIC by micro-injection of a fluorescent dye. The main limitation of such an experiment is that the micro-injection process takes up to 15 minutes to perform, at which time the explants would be exposed to ambient (20%) O_2. In the micro-injection experiments conducted for this thesis, this exposure did not result in the inhibition of GJIC in control explants maintained at 3% O_2 for 3 days. The only way to overcome this limitation would be to conduct the micro-injections within a specially designed oxygen-regulated enclosed culture hood, which is used by many laboratories that study the effects of oxygen on cell biology.

The results of the present study reveal the importance of gap junctions in trophoblast differentiation and hence overall placental development. In cases of abnormal placentation, such as preeclampsia, Cx40 may not be induced in EVT cells that reaggregate and approach maternal vessels to replace endothelial cells. Analogous to the inability of these deeply invaded EVT cells to upregulate α1 integrin and VE-cadherin, the lack of Cx40 would also render these cells less 'vascular-like', since Cx40 is the main endothelial connexin. To determine whether Cx40 plays a role in the etiology of preeclampsia or IUGR, placental bed biopsies from these pathological cases could be immunostained for Cx40 to see if EVT cells around maternal arteries are able to upregulate Cx40.

Human trophoblast stem cells are currently being isolated for long-term culture, providing an exciting future in trophoblast research. Mouse trophoblast stem cell lines have been developed and are currently being used to dissect mouse trophoblast differentiation (252).

With human trophoblast stem cell lines, connexin expression could be mapped during differentiation into invasive EVT cells. Connexins could also be transfected into these cells to determine the effects of specific connexins on trophoblast proliferation or invasion. There are definitely vast possibilities for the study of GJIC and human trophoblast function.

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