THE EXPRESSION AND ROLE OF INTEGRIN-LINKED KINASE DURING HUMAN TROPHOBLAST DIFFERENTIATION

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THE EXPRESSION AND ROLE OF INTEGRIN-LINKED KINASE DURING HUMAN TROPHOBLAST DIFFERENTIATION

PhD THESIS

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Abstract

A critical phase of placental development is the differentiation of cytotrophoblasts into invasive extravillous trophoblasts. Alterations of this process can lead to serious pregnancy pathologies such as preeclampsia and intrauterine growth restriction. Unfortunately it is poorly understood which physiological and molecular mechanisms are involved in the regulation of trophoblast differentiation.

Integrin-Linked Kinase (ILK) is a ubiquitous protein able to regulate outside-inside signaling by binding to integrins. Since ILK is overexpressed in tumors that become invasive, it was hypothesized that ILK is expressed in human placenta and that it is necessary for the differentiation of trophoblasts into invasive cells. To test the hypothesis, spatial and temporal patterns of ILK expression were studied in normal human and mouse placentas by immunohistochemistry, immunofluorescence analysis, and immunoblot analysis. In human placenta, ILK was present in cytotrophoblast and extravillous trophoblast in the first trimester (n=17), early second trimester (n=6) and at term (n=10). By comparison, ILK was present in the three layers of the mouse placenta: labyrinth, junctional zone, and trophoblast giant cells.

HTR8-SVneo cells were also used to determine the role of ILK in migration and invasion. When ILK function was impaired in HTR8-SVneo cells with a dominant negative form of ILK, migration and invasion were inhibited. Furthermore, HTR8-SVneo and human chorionic villous explant cultures were utilized to study the effect of the oxygen environment on ILK expression and activity. Exposure of HTR8-SVneo cells to a low oxygen environment produced a transient increase in the

expression of *ILK* mRNA, but did not affect the level of protein expression nor its kinase activity. In contrast to these results, placental explants showed lower expression of ILK under 3 % O_2 environment compared to placental explants exposed to 8 % and 20 % O_2. Placentas from preeclamptic patients also showed lower detection of ILK in the fetal capillaries compared to age-matched controls.

In total these novel results showed that ILK is present in human and mouse placenta in cells that invade the maternal uterus during pregnancy and that it plays a critical role in the migration and invasion of trophoblasts.

The results contribute to a better understanding of the mechanisms involved in the control of placental invasion and could assist in the production of future tools to improve the early diagnosis and treatment of gestational trophoblastic diseases such as preeclampsia.

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ACTB	Actin beta
AKT	AKT8 virus oncogene cellular homolog
ANK	Ankyrin
APE	Alanine-Proline-Glutamic acid triplet
AP-1	Activator protein 1
ATM	Ataxia-telangiectasia mutated protein
ATR	ATM-and Rad3-related protein
AV	Anchoring villi
BM	Basement membrane
BrdU	5-bromo-29-deoxyuridine
BSA	Bovine serum albumin
CAP	Contraction-associated protein
Cdc42	Cell division cycle protein 42 homolog
CK7	Cytokeratin 7
COX2	Cyclooxygenase 2
CSF-1	Colony stimulating factor-1
CSH1	Human chorionic somatomammotropin
CT	Cytotrophoblast
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
Cx	Connexin
DAPI	4',6-diamidino-2-phenylindole

Dec	Decidua
DEPC	Diethylpyrocarbonate
DFG	Aspartic acid-phenylalanine-glycine amino acid triplet
DFX	Desferrioxamine
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
dNTPs	Deoxyribonucleotides
DRH	Aspartic acid-Arginine-Histidine
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EGF	Epithelial growth factor
EGFP	Enhanced green fluorescent protein
EMT	Epithelial mesenchymal transition
EPC	Ectoplacental cone
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
EVT	Extravillous trophoblast
FA	Focal adhesion
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

fRBC	Fetal red blood cells
FV	Floating villi
GEF	Guanosine exchange factor
GLUT	Glucose transporter
GlyC	Glycogen cells
GSK3β	Glycogen synthase kinase 3 beta
HB-EGF	Heparin-binding epidermal growth factor
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-induced factor 1 alpha
HLA-G	Human leukocyte antigen G
hPGH	Human placental growth hormone
HRD	Histidine-Arginine-Aspartic acid triplet
HRP	Horseradish peroxidase
ICM	Inner cell mass
IF	Immunofluorescence
IFN-γ	Interferon gamma
IGF-BP	Insulin growth factor- binding protein
IGF-II	Insulin-like growth factor II
IHC	Immunohistochemistry
IL	Interleukin
ILK	Integrin-linked kinase
ILK-AP	Integrin-linked kinase-associated serine/threonine phosphatase 2C

ILK-E359K	Dominant negative form of integrin-linked kinase
ILK-WT	Integrin-linked kinase-wild type
ILK-S343D	Constitutively active form of integrin-linked kinase
ITGAV	Alpha v integrin
ITGA5	Alpha 5 integrin
ITGA5B1	Alpha 5 integrin-beta 5 integrin
IUGR	Intrauterine growth restriction
kDa	Kilodalton
KIR	Killer inhibitory receptor
Lab	Labyrinth
LDL	Low density lipoprotein
LIF	Leukemia-inhibiting factor
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
MHC	Histocompatibility complex
Mik1	Mitosis inhibitor kinase 1
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
αNAC	α Nascent polypeptide-associated complex
Nck-2	Non-catalytic region of tyrosine kinase adaptor protein-2
NF-κB	Nuclear factor-κB
NSCLC	Non-small-cell lung cancer

PAK	p21-activated kinase
PARP-1	Poly(ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide-dependent kinase 1
PE	Preeclampsia
PET	Polyethylene terephthalate
PFA	Paraformaldehyde
PH	Pleckstrin-homology domain
PINCH	Particular interesting new cysteine-histidine-rich protein
PI3K	Phosphoinositide 3 kinase
PIP3	Phosphatydylinositol (3,4,5)-triphosphate
αPIX	α-PAK interacting exchange factor
PKB	Protein kinase B
PlGF	Placental growth factor
Pl	Placental lactogen
Plf	Proliferin
PR	Progesterone receptor
qRT-PCR	Quantitative-Real time polymerase chain reaction
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family member A protein

Rictor	Rapamycin-insensitive companion of mTOR
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RRX	Rhodamine Red X
SCC	Squamous cell carcinoma
sEng	soluble endoglin
Smad	Small body size/ mothers against decapentaplegic
SDS	Sodium dodecyl sulfate
sFlt1	soluble Fms-related tyrosine kinase 1-VEGF receptor
SILAC	Stable isotope labeling with aminoacids in cell culture
ST	Syncytiotrophoblast
Sp	Spongiotrophoblast
TBST	Tris buffer saline Tween 20
TGC	Trophoblast giant cells
TGFβ	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
tIPP	ILK, PINCH and parvins ternary complex
TIRF	Total internal reflection fluorescence
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
uNK	Uterine natural killer
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

vCT	Villous cytotrophoblast
Vps15p	Vacuolar protein sorting 15p
VWF	von Willebrand factor
WKGRW	Tryptophan-Lysine-Glycine-Arginine-Tryptophan motif
ZBF	Zinc buffered fixative

CHAPTER 1

INTRODUCTION

1. 1 Human Placenta

The placenta is a remarkable transient organ present only in female mammals during gestation. It has several particular characteristics that make it unique. First, it is an organ formed by cells originating from two separate organisms in close relationship, the fetus and the mother who collaborate in a dynamic equilibrium to maintain the homeostasis of both. Throughout pregnancy, particularly at the beginning, cells derived from the trophectoderm of the implanting blastocyst in concert with cells from the maternal decidua combine to form the definitive placenta (Benirschke and Kaufmann 2000). During this process, fetal-derived cells invade the maternal uterus in a finely regulated manner. This characteristic in particular, makes the placenta an important model for identifying pathways and/or therapeutic strategies to treat cancer, a disease marked by uncontrolled invasiveness (King et al. 2000; Lala et al. 2002; Ferretti et al 2007). Also, in spite of the placenta being what immunologists consider a semi-allograft organ, it does not trigger a host-allograft rejection from the mother to the baby, confirming that the placenta has distinctive features (Creasy and Resnik 2000). The main functions of the placenta are to supply nutrients and oxygen to the fetus and to remove waste in the opposite direction. It also provides immune protection, endocrine and metabolic support to the developing fetus.

Thus, normal and adequate placental development is crucial for the well-being of both the baby and the mother. Abnormal formation of the placenta leads to serious pathologies such as preeclampsia and intrauterine growth restriction (IUGR) stressing the importance of the regulation of this process (Zhou et al. 1993; Kaufmann et al. 2003). A recent review of the literature showed that preterm birth in the USA increased from 9.5 % in 1981 to 12.7 % in 2005 (Goldenberg et al. 2008). Interestingly while the percentage of spontaneous preterm births have remained constant through the years and preterm birth due to premature rupture of the membranes have declined as time passes, much of the increase in the singleton preterm birth rate is explained by rising numbers of preterm birth initiated by the physician to prevent serious complications such as eclampsia (Goldenberg et al. 2008). Coincidently, the only treatment at the moment for preeclampsia is to deliver the baby when the symptoms appear, which makes preeclampsia one of the major reasons for that increase in prescribed preterm birth. A better understanding of the mechanisms involved in the control of placental invasion might improve the prevention of preeclampsia or provide tools for an early diagnosis or a better treatment. It might also help to understand the high numbers of miscarriages that at the moment have no identified explanation, and last but not least, it might also provide clues for mechanisms regulating cell invasion that could then lead to new cancer treatments and/or diagnosis.

1.1.1 Macroscopic Structure of the Placenta

The word *placenta* comes from the Latin for *cake*, from Greek *plakóenta/plakoúnta*, "flat, slab-like" (Liddell and Scott 2009), referring to its round, flat appearance in humans. Macroscopically, a delivered normal term placenta can be described as a disk-shaped, round, or ovoid structure. The blood cord is normally inserted near the center of the disk and the membranes are attached at the periphery. There is an enormous variability in placental size and shape but usually the normal placenta weighs between 400 g and 500 g at term and measures around 18 to 20 cm in diameter and approximately 2 cm thick (Benirschke and Kaufmann 2000). Some variations can be explained by racial differences, altitude, pathologic circumstances at implantation, diseases or maternal habits such as smoking. Absolute growth, as determined by DNA, RNA, and protein content, occurs in the placenta from fecundation until the 36^{th} week of gestation. Thereafter, proliferation does not normally occur and the placenta undergoes only maturational changes until the end of the gestation which in humans can be between 38 to 42 weeks long (Benirschke and Kaufmann 2000).

The fetal surface, facing the amniotic cavity, has a glossy appearance because of the intact epithelial surface of the amnion. The maternal uterine face of the placenta is opaque and has a rough texture, as it is a surface originating from laminar degenerative processes within the junctional zone that lead to the separation of the organ during labour. It is composed of a heterogeneous mixture of trophoblastic and decidual cells embedded into prevailing amounts of extracellular debris, fibrinoid and blood clot (Bernischke and Kaufman 2000). The maternal surface of the placenta is divided into lobes (or cotyledons) by an incomplete system of grooves which

internally correspond to septa that trace the lobules' borders (Benirschke and Kaufmann 2000). When placentas are injected with a radiopaque medium they show that each lobe is occupied by one to three villous trees (Benirschke and Kaufmann 2000) which are the structural and functional units of the placenta as will be explained in detail in the next section.

1.1.2 Microscopic Structure of the Placenta

The placenta only forms after an ovum has been fertilized and the embryo has been implanted in the mammalian uterus. Fertilization occurs in the fallopian tube within 24 to 48 h after ovulation and the initial steps of development, from fertilized ovum to a morula stage of 12 to 16 cells, occur as the embryo passes through the fallopian tube. After arriving to the uterine cavity and before implantation, a fluid-filled inner cavity appears within the mass of cells and marks the transition from morula to blastocyst (Theiler 1989; Enders et al. 1990; Sadler 2006). This is accompanied by cellular differentiation: the surface cells become trophectoderm while an inner cell mass is formed and becomes the precursor of the embryo proper (Johnson and Ziomek 1981). Within 72 h after entering the uterine cavity, the embryo hatches from a non-adhesive coating called the zona pellucida and exposes the trophectoderm (Red-Horse et al. 2004). Implantation is then dependent on a set of complex interactions between the hormonally primed uterus and the mature blastocyst (Enders 2000). Some molecules that are involved in endometrial receptivity include Leukemia Inhibiting Factor (LIF) (Cullinan et al 1996; Norwitz et al 2001), adhesion

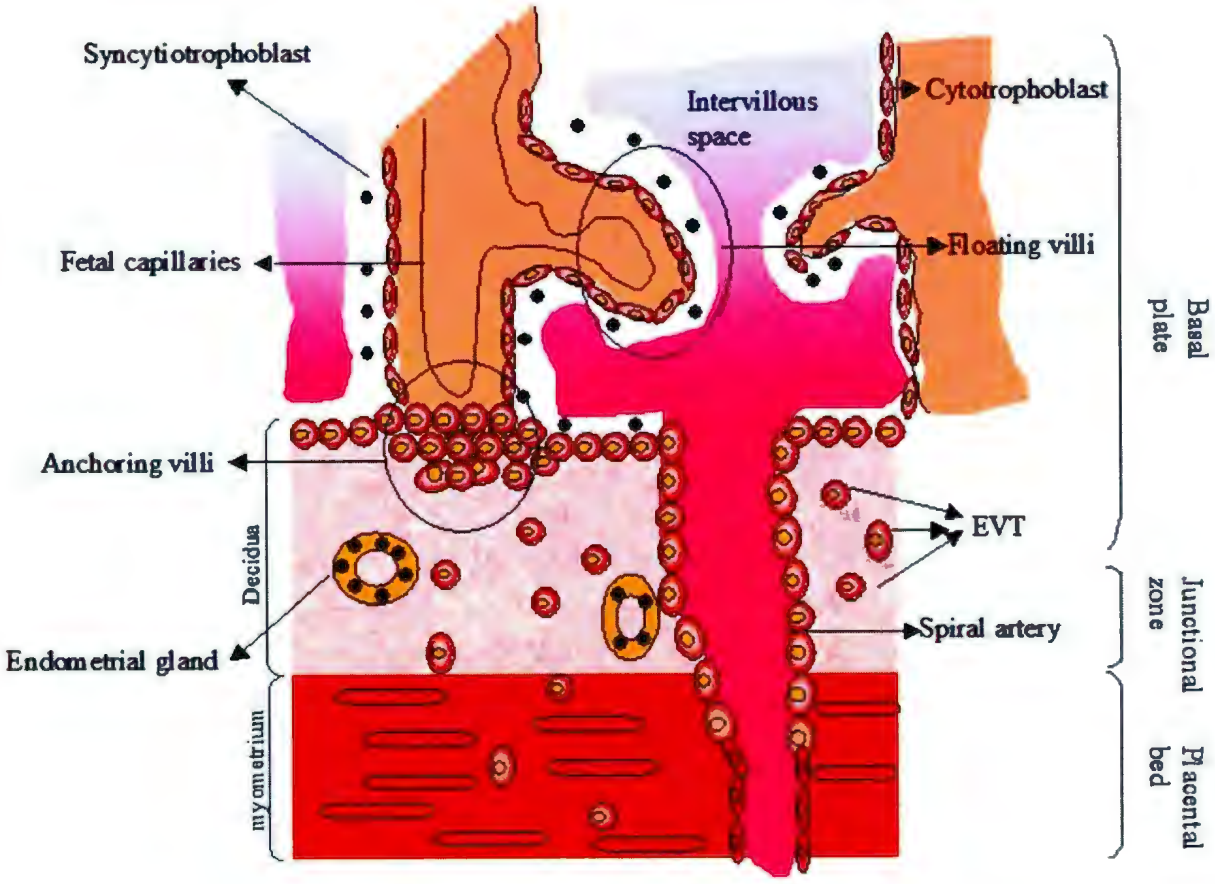
molecules, such as integrins (Lessey and Castelbaum 2002), and proteins called mucins that have high sugar content and cause an increase in the expression of oligosaccharide receptors on the surface of endometrial epithelial cells (Lagow et al. 1999). Embryos at/or near the implantation stage express Epidermal Growth Factor Receptors (EGFR) and heparan sulfate proteoglycans, both of which interact with epidermal growth factor–like ligands. Proper implantation also depends on the differentiation of the trophectoderm into the different trophoblast lineages that will form the placenta. The term "trophoblast" was introduced by A.A.W. Hubrecht, a Dutch scholar, at the end of the 19th century (Hubrecht 1899, cited by Boyd and Hamilton 1966) to designate those cells derived from the blastocyst that do not contribute to the embryo but are essential for its nourishment (Norwitz et al. 2001; Red-Horse et al. 2004; Gude et al. 2004). Immediately after implantation, trophoblasts derived from the trophectoderm differentiate and invade the maternal uterine wall. By the 10th day after conception, the blastocyst is completely embedded in the stromal tissue of the uterus, the uterine epithelium has regrown to cover the site of implantation, and the trophectoderm has differentiated into an outer layer of syncytiotrophoblast underlied by a mononuclear layer of cytotrophoblasts (CT) forming a primitive placenta with primary villi (Pijnenborg et al. 1981; Pijnenborg 1988; Benirschke and Kaufmann 2000). The primary villi are then infiltrated by mesenchyme to form secondary villi. The definitive villi (tertiary villi), which are the structural and functional units of the fetomaternal interface, become apparent as early as 21 days of pregnancy (Benirschke and Kaufmann, 2000). The trophoblasts, coordinated with the mesenchyme and capillaries, proliferate and differentiate to

organize themselves into floating villi and anchoring villi (Fig 1.1). These tree-like arranged placental villi arise from the chorionic plate and protrude into the intervillous space that is filled with maternal blood. The surface of the floating villi is composed of an outer continuous layer of villous syncytiotrophoblast beneath which there is a layer of villous cytotrophoblasts (vCT). The stroma of the villi is composed of the fetal vessels which are embedded in a mixture of, macrophages, connective tissue cells and connective tissue fibers and it is separated from the vCT by a basement membrane (BM). The ramifications of the villous tree can be subdivided into segments that differ mainly in caliber, stromal structure, vessel structure and relative position within the villous tree and these characteristics define five villous types (Benirschke and Kaufmann, 2000). Although the description of each type of villi is beyond the scope of this thesis, it is important to note that as a consequence of presenting different types of villi, human placentas studied under the microscope may show heterogenous groups of villi with differences in number and structure of fetal blood vessels and diameter.

1.1.3 Overview of Trophoblast Differentiation

During early placentation, two fundamental pathways of trophoblast differentiation take place: the fusion and invasion pathways. In the fusion pathway, polarized stem vCT in floating villi proliferate and fuse with existing syncytiotrophoblast to maintain the multi-nucleated layer (Huppertz et al. 2002; Baczyk et al. 2006; Huppertz and Gauster 2011). The vCT in the floating villi

Figure 1.1 Schematic drawing of the human placenta. The arrows point at the main structures forming the floating and anchoring villi. EVT: extravillous trophoblast (adapted from Gray's Anatomy of the Human Body. Gray H, 20th Ed).


Syncytiotrophoblast
Intervillous space
Cytotrophoblast
Fetal capillaries
Floating villi
Anchoring villi
EVT
Decidua
Endometrial gland
Spiral artery
myometrium
Basal plate
Junctional zone
Placental bed

represent the proliferating stem cells for the syncytiotrophoblast. The syncytiotrophoblast main functions are the exchange of gas and nutrients as well as the synthesis of essential hormones.

In the invasion pathway, polarized stem CT cells in anchoring villi tips migrate off the villous basement membrane, penetrate through the syncytiotrophoblast to form columns of non-polarized extravillous trophoblast (EVT) cells which connect the embryo to the uterine wall (Castellucci et al. 2000; Georgiades et al. 2002; Pollheimer and Knofler 2005). These anchoring villi attach the placenta firmly to the uterus and give rise to both the EVT that invade the decidua (interstitial EVT), and those that remodel the maternal arterioles, endovascular EVT. The formation of anchoring villi is accompanied by changes in the synthesis, degradation, and spatial distribution of extracellular matrix (ECM) proteins, and the specific spatial/temporal expression of adhesion molecules (Damsky et al. 1992; Damsky et al. 1994; Aplin et al. 2006; Aplin et al. 2009). Proximal cell columns of EVT cells in the villous tip are proliferative, rounded, cohesive cells that are marked by the high expression of $\alpha 6\beta 4$ integrin, E-cadherin and laminin (Damsky et al. 1992). The more distal cells that are interstitial and endovascular EVT cells have differentiated into isolated, highly invasive, pleomorphic cells that express $\alpha 5\beta 1$ and $\alpha 1\beta 1$ integrins, fibronectin and type IV collagen (Damsky and Werb 1992).

The transformation of the arterioles produces dilated uterine arterioles that are unresponsive to maternal vasomotor control (Gude et al. 2004) ensuring that enough blood supply reaches the intervillous space for the proper exchange with the fetus. In the non-pregnant state, the uterine vessels carry < 1 % of the maternal

cardiac output as it only has to maintain a uterus that weighs only around 50 g. At term, these same vessels must support a uterus, placenta and fetus that can weigh up to 5000 g altogether (Kliman 2000). To adapt to this demand, instead of increasing the amount of vessels which would increase linearly the amount of blood flow, the maternal arteries are transformed into wider vessels that do not respond to vasoactive compounds increasing the blood flow exponentially thus ensuring that the fetus will receive enough blood (Kliman 2000). Another specialization for the optimization of exchange is the presence of microvilli at the surface of the syncytiotrophoblast that increase the effective surface area for nutrients and gas exchange (Benirschke and Kaufmann 2000). The factors that regulate trophoblast differentiation will be discussed in detail in Section 1.1.5.

1.1.4 Human Placental Functions

The main functions of the placenta are transport, immune protection and endocrine support of the fetus.

1.1.4.1 Transport of Gases and Nutrients

1.1.4.1.1 Transport of Gases. The lungs are not functional during fetal life so gas exchange occurs through the placenta by simple diffusion. The direction of oxygen movement from the mother to the fetus is facilitated by the higher

concentration and affinity for oxygen of fetal hemoglobin when compared to maternal hemoglobin (Bauer and Rathschlag-Schaefer 1968; Tyuma and Shimizu 1969; Bunn 1981). Interestingly, in pregnant women who smoke the carbon monoxide in the cigarette competes with oxygen for the hemoglobin and transforms the fetal hemoglobin into carboxyhemoglobin which has very low affinity for oxygen exposing the fetus to a transient hypoxia (Soothill 1996). This contributes partially to the fetal growth restriction in fetuses born from women that smoke and that is highly prevalent in these patients.

1.1.4.1.2 Transport of Nutrients. The nutrient transfer capacity of the placenta, which is dependent on adequate placental development, plays a critical role in fetal growth (Barry et al. 2008). Most of the nutrients use active transporters and the passage through the placenta is essential for the viability of the fetus. For example, glucose is the main carbohydrate transported across the placenta from the mother to the fetus. As the fetal gluconeogenesis metabolic enzymes are not well developed, the glucose must be derived from the maternal circulation. Transport of glucose across the placenta is via protein-mediated facilitated diffusion and a number of glucose transporters (GLUT) are involved, such as GLUT 1 and GLUT 3 (Baumann et al. 2002). Intrauterine growth restriction (IUGR), usually a consequence of placental malfunction, is associated with altered glucose metabolism with an increase of GLUT1 expression at the placental interface (Langdown and Sugden 2001).

Transport of amino acids to the fetus during pregnancy occurs via the microvilli and basal membranes of the syncytiotrophoblast and is mediated by

specific active transporters. Using functional criteria such as substrate specificity and sodium dependence, approximately 15 different transport systems have been identified in the human placenta (Jansson 2001; Jansson et al. 2009). Furthermore, the actual absolute placental amino acid transport is reduced in cases of fetal growth restriction (Regnault et al. 2005).

Lipids include free fatty acids, triacylglycerols, phospholipids, glycolipids, sphingolipids, cholesterol esters and fat soluble vitamins among others. Both free fatty acids and triacylglycerols can readily cross the membranes of the syncytiotrophoblast by simple diffusion. Once they reach the cytoplasm of the placental trophoblast, they can bind to cytoplasmic proteins, they can be transported out of the trophoblast, or alternatively they can be oxidized or esterified. Cholesterol is derived from maternal blood via an interaction of circulating low density lipoprotein (LDL) with LDL receptors on the microvillous membrane of the syncytiotrophoblast followed by internalization of LDLs by receptor-mediated endocytosis (Gude et al. 2004).

1.1.4.2 Immune Function of the Placenta

The placenta is considered a semiallograft as it contains half of the genes from the father in direct contact with the mother. Interestingly, there is no immune response triggered by these "foreign" molecules. In 1953 Medawar was the first to suggest that the semi-allogeneic fetus is able to survive because either there was an anatomical separation between the mother and the fetus or that there was a functional

suppression of maternal lymphocytes (Medawar 1953; Veenstra van Nieuwenhoven et al. 2003). It is now known that there is no physical separation between the mother and the fetus as various cells such as syncytiotrophoblast and EVTs are in direct contact with maternal blood thus with maternal immune cells. There is, however, a lack of antigen stimulation of maternal lymphocytes (Veenstra van Nieuwenhoven et al. 2003), since the syncytiotrophoblast lacks both class I and II major histocompatibility complex (MHC) molecules and the fetal EVT do not express major MHC Ia antigens, which are responsible for the rejection of allografts in humans (Veenstra van Nieuwenhoven et al. 2003). The EVT only express human leukocyte antigen (HLA) G and E which are non-polymorphic members of the class I histcompatibility complex. HLA G and E are thought to be involved in the resistance of EVT cells to lysis by uterine natural killer (uNK) cells via direct binding with killer inhibitory receptor (KIR) on the uNK (Pazmany et al. 1996; Munz et al. 1997; Rieger et al. 2002). As for the immune suppresion in Medawar's theory, there is not a general suppression but a change in lymphocyte function. There is a shift away from type 1 cytokine production with a decrease in the type 1/type 2 cytokine ratio which is beneficial for normal pregnancy. Type 1 cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα) are harmful for pregnancy because they inhibit embryonic and fetal development (Chaouat et al. 1990) and terminate pregnancy when injected in pregnant mice. The decreased ratio of type 1/type 2 cytokine production may be explained in different ways. First, hormones such as progesterone and estrogen may directly affect lymphocyte production towards type 2 cytokines (Piccinni et al. 1995). Also, syncytiotrophoblasts express indolamine dioxygenase an

enzyme which functions in the catabolism of tryptophan and indirectly suppresses maternal T-cell activity by tryptophan deprivation (Schrocksnadel et al. 1996; Munn et al. 1998; Kudo et al. 2001).

There are other mechanisms that the trophoblast may use to escape the maternal immune system. One way is by the expression of apoptosis-inducing ligands (Veenstra van Nieuwenhoven et al. 2003). The expression of Fas Ligand (FasL) was observed in syncytiotrophoblast, in vCT and EVT, as well as the expression of the receptor Fas was found on decidual leukocytes suggesting that FasL expression on trophoblasts might be a mechanism protecting the trophoblasts against activated leukocytes (Runic et al. 1996; Uckan et al. 1997; Kauma et al. 1999, Veenstra van Nieuwenhoven et al. 2003). Another apoptosis-inducing pathway such as binding of the TNF-related apoptosis-inducing ligand (TRAIL) to its receptor (TRAIL-R) may play a role in immunomodulation during pregnancy as TRAIL is expressed on trophoblasts (Phillips et al. 1999; Phillips et al. 2001). With these ligands, trophoblasts cells can induce apoptosis of activated immune cells if necessary and protect the placenta and the fetus from a harmful immune response (Veenstra van Nieuwenhoven et al. 2003). Furthermore, the trophoblasts express CD46, a complement receptor that binds C3b and C4b, preventing complement mediated damage to the trophoblast and villous mesenchyme (Holmes and Simpson 1992). Trophoblasts also express cytotoxic T lymphocyte associated antigen 4 (CTLA-4) which binds and inactivates activated lymphocytes by binding to CD80 and CD86 (Heikkinen et al. 2004). In summary, the placenta does not present antigens to the

immune system and in case an immune response is triggered, the trophoblasts have all the machinery required to stop the response *in situ* so there is no damage to the fetus.

1.1.4.3 Endocrine Functions of the Placenta

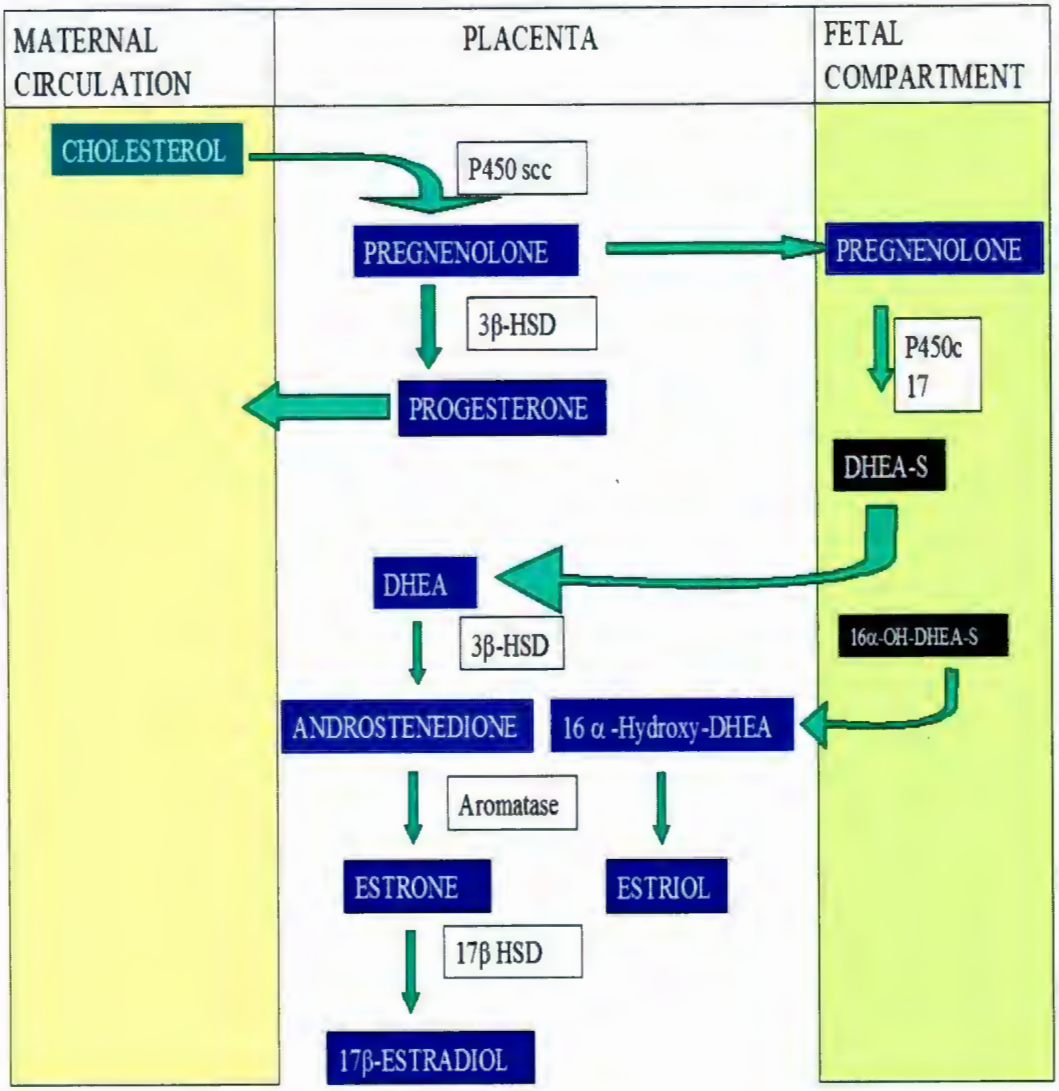
Endocrine, paracrine and growth factors produced by the placenta include: estrogens (produced in conjunction with the fetal adrenal gland and possibly fetal liver), progesterone, human chorionic gonadotropin (hCG), human chorionic somatomammotropin (CSH1, originally called placental lactogen), human placental growth hormone (hPGH), growth factors such as epidermal growth factor (EGF), insulin-like growth factors I and II (IGF-I and IGF-II), and platelet-derived growth factor (PDGF), as well as cytokines, chemokines, eicosanoids and related compounds, vasoactive autocoids, and many others like corticotropin-releasing hormone. Discussion of all of these factors is beyond the scope of this thesis; however, a short description of hCG and the steroids estrogen and progesterone is warranted.

1.1.4.3.1 Human Chorionic Gonadotropin: hCG is one of the earliest pregnancy-associated peptides secreted by the cells of the placenta. After implantation, hCG is produced principally by the syncytiotrophoblast. It can be detected in the serum or urine as early as 7 to 8 days before expected menses and reaches a peak of 100,000 mU/ml by the 9^{th} or 10^{th} week of gestation (Creasy and Resnik 2000). The major biological role of hCG during early pregnancy is to maintain the corpus luteum until the placenta starts synthesizing progesterone (Creasy and Resnik 2000). During this phase of pregnancy if progesterone actions are blocked by

a competitive antagonist, such as mifepristone, or the corpus luteum is removed before 9-10 weeks, pregnancy cannot be maintained unless progesterone supplementation is provided. After the 10^{th} week of gestation the corpus luteum disappears and the major source of progesterone is the placenta (Creasy and Resnik 2000). Levels of hCG in serum drop down to 5,000-10,000 mU/ml and remain constant until the end of the pregnancy (Cole 1997).

1.1.4.3.2 Progesterone and Estrogens: Progesterone is synthesized within the syncytiotrophoblast mainly from maternal cholesterol (Fig 1.2). Ninety percent of the progesterone is secreted into the maternal compartment and the rest is secreted into the fetal compartment where it is converted into fetal adrenal steroids, cortisol and dehydroepiandrosterone (DHEA). In contrast to other steroidogenic organs, the placenta does not express cytochrome P450 17-α-hydroxylase-17:20 lyase (P450c17) and cannot transform progesterone into androgens. Therefore placental estrogen depends on androgen precursors (DHEA) produced by the maternal adrenal gland and by the expanded zone of the fetal adrenal gland. The biosynthesis of estrogens demonstrates the interdependence of the fetus, the placenta and the mother (Fig 1.2). During pregnancy a woman produces more estrogen than a normal woman could produce in more than 150 years (Tulchinsky et al. 1972). The major estrogens formed in the placenta are estriol, estradiol, estrone and esterol. The major androgenic precursor to placental estrogen formation is DHEA sulfate (DHEA-S), which is the major androgen produced by the fetal adrenal cortex. DHEA-S transported to the placenta is cleaved by sulfatase to form free unconjugated DHEA and is then aromatized by placental aromatase cytochrome P450 to estrone and 17β-estradiol.

Figure 1.2 Progesterone and estrogens synthesis during pregnancy. Progesterone and estrogens are produced by the placenta with precursors derived from the maternal and fetal circulation. P450scc, P450 cholesterol side-chain; p450c17, P450 17α-hydroxylase/17--20 lyase enzyme, 3β-HSD, 3 beta hydroxysteroid dehydrogenase; 17β-HSD, 17 beta hydroxysteroid oxidoreductase; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.


MATERNAL CIRCULATION
PLACENTA
FETAL COMPARTMENT
CHOLESTEROL
P450 scc
PREGNENOLONE
PREGNENOLONE
3β-HSD
P450c 17
PROGESTERONE
DHEA-S
DHEA
3β-HSD
16α-OH-DHEA-S
ANDROSTENEDIONE
16 α -Hydroxy-DHEA
Aromatase
ESTRONE
ESTRIOL
17β HSD
17β-ESTRADIOL

1.1.4.3.3 Role of Estrogens and Progestins: Even before implantation a normal level of these hormones is important as progesterones and estrogens induce the formation of a receptive uterus which is critical for implantation. Studies with transgenic approaches showed that uterine expression of progesterone receptor (PR) A and estrogen receptor (ER) α, but not PR B or ER β, are required for implantation (Conneely et al. 2002; Hewitt and Korach 2002). In mice, a very narrow range of estrogen concentrations determines the duration of the window of receptivity, i.e., the period of time during which the uterus is able to support implantation (Ma et al. 2003). Estrogen also has effects on the blastocyst, as its 4-hydroxy catechol metabolite mediates blastocyst activation, a requisite step in implantation (Paria et al. 1998). Once implantation has occurred progesterones are important in maintaining the uterine quiescence during pregnancy by actions on uterine smooth muscle. It is proposed that progesterone may be acting by limiting the production of stimulatory prostaglandins and inhibiting the expression of contraction-associated protein (CAP) genes (ion channels, oxytocin and prostaglandin receptors and gap junctions) within the myometrium (Norwitz et al. 1999; Challis et al. 2000). Administration of exogenous progesterone at term not only blocks the expression of CAP genes, but also blocks the onset of labor (Lye and Porter 1978). Even in humans, where there is no evidence of a fall in maternal plasma or uterine progesterone, administration of the PR antagonist RU486 leads to increased uterine activity and induction of labor (Avrech et al. 1991). Progesterone also inhibits prostaglandin production presumably contributing to uterine quiescence and delaying cervical ripening (Andersson et al. 2008). Progesterone also plays an important protective role by inhibiting T-

lymphocyte mediated reactions protecting the baby from an immune response (Grossman 1985). It also plays a role in homing of uNK cells and up-regulates HLA-G gene expression, the ligand for various NK inhibitory receptors. At high concentrations, progesterone is a potent inducer of Th2-type cytokines as well as of LIF and monocyte-colony stimulating factor (M-CSF) production by T cells (Mori et al 1977; Arck et al. 2007). All these functions point at progesterone as one of the critical hormones during pregnancy not only to prevent labour but alos to protect the fetus from an immunological rejection.

Estrogens have a broad variety of functions in the physiology of pregnancy as they stimulate the ripening of the cervix (Andersson et al. 2008) and stimulate the increase in prostaglandins necessary for initiation of labour (Challis 1971; Challis et al. 2000). Apart from the important role that estrogens play during labor, they increase uterine blood flow and prepare the breast for lactation (Pepe and Albrecht 1995).In 1967, Pinto et al. (1967a; 1967b) examined the role of estrogens in human parturition by administrating a large amount of 17β-estradiol (200 mg intravenously in 1 h) to non-laboring pregnant women at term. They found that estradiol treatment increased uterine contractility within 4–6 h and accelerated the time to delivery. They also showed that the administration of progesterone rapidly induced a block in the estrogen effect (Pinto et al. 1967 b). Studies in rats and sheep also demonstrated that treatment with estradiol at mid-gestation induces preterm labor (Dawe et al. 1982; Wu et al. 2004). In humans (and higher primates) circulating estrogens increase at around mid-gestation and continue to rise gradually until birth, in contrast with most of the non-primates that show a sharp increase in estrogen concentration before labor

onset (Tulchinsky et al. 1972; Boroditsky et al. 1978; Mesiano 2007). This has led to the concept that estrogen activation in human parturition, as with progesterone withdrawal, is mediated at the functional level, by an increase in myometrial estrogen responsiveness. Thus, for most of pregnancy, the human myometrium appears to be refractory to estrogens at least in terms of CAP gene expression. This lack of response of the myometrium to estrogens is likely due to very low levels of ER α and ER β. Importantly, ER α expression is low in non-laboring term myometrium and increases in association with labour onset, suggesting that functional estrogen activation is mediated by increased ER α expression (Mesiano et al. 2002) during parturition.

1.1.5 Placental Development Regulation

During placental formation cells derived from the trophectoderm invade the decidua. This is a finely regulated mechanism of invasion as it is ultimately restricted to the inner third of the myometrium in humans and only to the decidua in rodents (Pijnenborg et al. 1980; Georgiades et al. 2002). Abnormally shallow invasion leads to serious pathologies such as preeclampsia and IUGR stressing the importance of the regulation of this process (Zhou et al. 1993; Kaufmann et al. 2003). Excessive invasion can lead to deficient development of the decidua with abnormally firm attachment of the placenta directly onto the myometrium (placenta increta), or to invasion through the myometrium to the uterine serosa and even into adjacent organs (placenta percreta) (Norwitz et al. 2001). It is not completely understood which

physiological and molecular mechanisms are involved in the regulation and control of trophoblast differentiation, but the local oxygen environment (Genbacev et al. 1997; Caniggia et al. 2000a), paracrine signals secreted by decidual cells, autocrine control from trophoblast-derived growth factors and interaction with different ECM components are likely to be involved (Damsky et al. 1992; Kilburn et al. 2000; Leach et al. 2004; Pollheimer and Knofler 2005). The action of the main regulators will be discussed below.

1.1.5.1 Oxygen as a Regulator of Trophoblast Differentiation

Due to the nature of the tissue, most of what is known about the morphological aspects of early placental formation has been extrapolated from animal models (Kliman 2000). Examination of monkey implantation sites has revealed that trophoblasts begin migrating down into the maternal arteries as early as 10 days after fertilization and at 14 days many of the spiral arteries, but not the veins, are occluded (Blakenship et al. 1993). Studies in humans using histochemical and ultrasonographic approaches suggested that there is in fact trophoblast plugging of the maternal spiral arteries until 12 weeks of gestation (Pjinenborg et al. 1982). More evidence came from studies that perfused hysterectomy specimens from pregnancies at week 8, 9 and 13 weeks of gestation with barium sulfate and examined them roentgraphically (x-ray photography). Spatial reconstruction of the uteroplacental network clearly showed a continuous trophoblastic shell at the level of the uterine-intervillous interface and no direct entry of the contrast medium inside the intervillous space at weeks 8 and 9.

These findings showed that a very limited amount of maternal blood reaches the intervillous space during the first trimester of gestation (Hustin and Schaaps 1987; Jaffe et al. 1997). Although the evidence was very strong, the concept that the placenta had low regional oxygen tension in the first trimester was further confirmed by elegant experiments done by Rodesch et al. (1992) and Jauniaux et al. (1999, 2001). By directly measuring placental oxygen partial pressure with an oxygen sensing probe they showed a significant increase in oxygen tension from about 25 mm Hg to 54 mm Hg at around week 10 of gestation. These experiments demonstrated that maternal blood flow into the intervillous space is only established at around the end of the first trimester (weeks 10 -12). Interestingly this is the time when the placenta develops enhanced mechanisms for protection against oxidative stress (Watson et al. 1998). So it is critical that maternal blood flow to the embryo be limited very early in gestation to protect the conceptus from excessively elevated oxygen levels that would lead to an increased production of harmful reactive oxygen species (Rodesch et al. 1992).

Virtually every cell type is capable of sensing a lack of oxygen and eliciting an adaptive response. A well recognized transcription factor associated with physiological responses to hypoxia is hypoxia-inducible factor 1 (HIF-1) identified as a mediator of oxygen tension effects in many mammalian systems (Semenza 1998). HIF-1 regulates changes in gene expression, including the transcription of specific genes that will help sustain the supply of oxygen to tissue cells during oxygen deprivation (De Marco and Caniggia 2002). Some examples of proteins expressed in response to low oxygen are vascular endothelial growth factor (VEGF) that is

involved in angiogenesis (Shweiki et al. 1992), erythropoietin that induces hemoglobin synthesis (Goldberg et al. 1988) or endothelin that is a vasoactive compound (Minchenko and Caro 2000). The adaptive response to low oxygen also includes the metabolism of glucose (Shetty et al. 1993; Semenza 2000) with increased expression of GLUT1, a glucose transporter.

There are extensive studies showing that trophoblast cells become highly proliferative in low oxygen tensions and as soon as they are cultured in 8 % or 20 % oxygen they differentiate into invasive trophoblast cells able to migrate and invade Matrigel while expressing HLA-G and matrix metalloproteinase (MMP) 9, markers of the invasive phenotype (Genbacev et al. 1996; Genbacev et al. 1997; Caniggia et al. 2000a). Early studies on the effect of oxygen on trophoblast differentiation showed that when anchoring villi from early gestation (6 to 8 weeks) were explanted onto an ECM substrate under standard conditions (20 % O_2 or 98 mm Hg) the cell columns were visible and kept the normal architecture, but did not incorporate 5-bromo-29-deoxyuridine (BrdU), a thymidine analog. The anchoring villi that were maintained in a hypoxic atmosphere (2% O_2 or 14 mm Hg) showed prominent cell columns and the nuclei of many of the CT in these columns incorporated BrdU (Genbacev et al. 1997). These results suggested that at low concentrations of oxygen the trophoblast cells proliferate while at 20 % oxygen (high levels) the cells do not proliferate but instead invade the artificial matrix. Concomitant with these results, when villous explants were subjected to antisense-induced inhibition of HIF-1α to simulate the effect of an increase in oxygen tension, the expression of transforming growth factor β (TGFβ)-3 was inhibited and EVT outgrowth and invasion were stimulated as indicated by the

expression of invasive markers such as MMP2 and MMP9, switching of the expression of integrins on the surface of the trophoblasts and downregulation of proliferation markers such as Ki67 (Rodesch et al. 1992; Caniggia et al. 2000b). These elegant experiments suggest that oxygen *per se* is an important factor regulating the differentiation of trophoblasts, inhibiting the proliferative phenotype of these cells and stimulating the invasive transformation of EVT necessary for normal placental development.

1.1.5.2 Autocrine and Paracrine Control of Trophoblast Invasion

During normal human pregnancies interstitial or endovascular invasion does not occur beyond the decidua and the first third of the underlying myometrium so the extent of trophoblast invasiveness must be precisely controlled by trophoblast-derived as well as maternal factors in a time- and distance-dependent manner (Graham and Lala 1992; Lala and Hamilton 1996; Bischof et al. 2000; Knofler 2010). Those observations are confirmed by histologic evaluation of ectopic pregnancies (Paterson and Grant 1975), in which trophoblasts are dramatically more invasive and destructive, suggesting once more that local factors in the decidua or endometrium inhibit trophoblast invasion (Creasy and Resnik 2000). Growth factors and the different components of the ECM affect trophoblast behaviour either promoting or inhibiting their proliferation, migration, invasion and survival. Numerous growth factors have been identified at the fetal-maternal interface controlling the proliferative

Table 1.1 Growth factors that regulate trophoblast proliferation.

EVT, extravillous trophoblast; vCT, villous cytotrophoblast; "+" stimulates; "-" , inhibits.

GROWTH FACTOR	CELL ORIGIN	EFFECT	REFERENCE
EGF	EVT	+	Maruo et al 1995
TGFα	EVT	+	Lysiak et al 1993
Amphiregulin	EVT	+	Lysiak et al 1995
CSF-1	Decidua, vCT and EVT	+	Hamilton et al 1998
Angiotensin II	EVT	+	Ino et al 2003
VEGF	EVT	+	Cha et al 2001
PlGF	EVT	+	Athanassiades and Lala 1998
TGFβ	Decidua	-	Graham and Lala 1992

EGF, epidermal growth facto; TGFα, transforming growth factor alpha; CSF-1, colony stimulating factor-1; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; TGFβ, transforming growth factor beta.

Table 1.2 Growth factors that regulate trophoblast migration or invasion.

EVT, extravillous trophoblast, "+", stimulates; "-", inhibits.

GROWTH FACTOR	CELL	EFFECT	REFERENCE
IGF-II	Decidua	+	Irving and Lala 1995
Endothelin	EVT and decidua	+	Chakraborty et al 2003
EGF	EVT	+	Bass et al 1994, Qiu et al 2004
HGF	Decidua	+	Cartwright et al 2002
HB-EGF	Decidua cell, EVT	+	Leach et al 2004
Activin A	Decidua cell	+	Cannigia et al 1997
TGFβ	Decidua cell	-	Graham and Lala 1992, Cannigia et al 1999
Endostatin	Decidua cell	-	Pollheimer et al 2011
Kisspeptin 10	Decidua cell	-	Bilban et al 2004; Pollheimer and Knofler 2005
sFtl-1	endothelium	-	Clark et al 1998
sEng	endothelium	-	Venkatesha et al 2006

IGF-II, insulin-like growth factor II; EGF, epidermal growth factor; HGF, hepatocyte growth factor; HB-EGF, heparin-binding epidermal growth factor; TGFβ, transforming growth factor; sflt-1, soluble fms-related tyrosine 1-VEGF receptor; sEng, soluble endoglin.

as well as invasive capacity of trophoblasts (Lala and Hamilton 1996; Bischof et al. 2000) (Tables 1.1 and 1.2). Different decidual cell types which are in close contact to interstitial EVT (i.e. decidual stromal cells, uNK cells and macrophages) are thought to regulate these processes in a paracrine manner. In addition, EVT express multiple ligands and hormones as well as their receptors indicating autocrine control (Bischof et al. 2000; Lala and Chakraborty 2003). Throughout gestation, growth factors such as EGF (Maruo et al. 1995), transforming growth factor-α (TGF-α), amphiregulin (Lysiak et al. 1993; Lysiak et al. 1995), colony stimulating factor-1 (CSF-1) (Hamilton et al. 1998), VEGF and placental growth hormone (PlGF) (Athanassiades and Lala 1998) are abundantly secreted from diverse cell types of the fetal-maternal interface including EVTs and were shown to promote proliferation of trophoblasts. In addition, in choriocarcinoma cells lines, angiotensin II and VEGF modulated cell proliferation through extracellular signal regulated kinase (ERK)-dependent signalling (Cha et al. 2001; Ino et al. 2003).

Other growth factors also promote migration and/or invasion. For example, IGF-II produced by trophoblasts (Irving and Lala 1995) promoted migration via inhibition of adenyl-cyclase and stimulation of mitogen-activated protein kinase (MAPK) in EVT (Hamilton et al. 1998; McKinnon et al. 2001). Furthermore, insulin growth factor binding protein (IGF-BP) produced by decidual cells, bind to RGD-binding sites of α5β1 integrin on EVT stimulating focal adhesion kinase (FAK) and MAPK which induced attachment to the ECM (Gleeson et al. 2001). Treatment of the cells with a specific MAPK/ERK kinase kinase (MEK) inhibitor reduced basal trophoblast migration and abolished the effects of IGF-II and IGFBP-1 on ERK1/2

phosphorylation, inhibiting the migration of the trophoblasts (Gleeson et al. 2001; McKinnon et al. 2001). Similarly, endothelin, produced by trophoblasts and decidua cells (Chakraborty et al. 2003) and EGF promoted HTR8-SVneo cell migration which was accompanied by activation/phosphorylation of ERK1 and ERK2 (Chakraborty et al. 2003; Qiu et al. 2004). Hepatocyte growth factor (HGF) promotes migration of SGHPL-4 cells, a trophoblast-derived cell line, via phosphoinositide 3 kinase (PI3K) (Cartwright et al. 2002). Another growth factor promoting migration of isolated first trimester trophoblasts is EGF (Bass et al. 1994). Qiu et al. (2004) showed that EGF induced both ERK and protein kinase B (PKB)/AKT8 virus oncogene cellular homolog (AKT) phosphorylation whereas EGF-activated cell migration could be blocked by inhibiting either MAPK or PI3K. In addition rapamycin, which specifically blocks mammalian target of rapamycin (mTOR), decreased phosphorylation of the 70 kDa S6 kinase and migration of the cells. HGF (Cartwright et al. 2002) and heparin binding-epidermal growth factor (HB-EGF) (Leach et al. 2004) also induce differentiation of trophoblasts into the invasive phenotype. Decidual cells also produce a variety of inhibitory proteins such as TGFβ family members, IFN-γ, endostatin, kisspeptin-10 or TNF-α to fine-tune and limit the extent of trophoblast invasion (Bauer et al. 2004; Bilban et al. 2004; Pollheimer and Knofler 2005; Lash et al. 2006). Signalling proteins of the small body size/ mothers against decapentaplegic (Smad) family are the downstream effectors of the TGFβ superfamily (TGFβ, activins, nodal, bone morphogenetic proteins) (Schiller et al. 2004). TGFβ factors were amongst the first identified regulators of invasive trophoblast differentiation since they inhibited proliferation of first trimester

trophoblasts (Graham and Lala 1992). Furthermore, TGFβ exerts anti-invasive effects on trophoblasts by increasing tissue inhibitors of metalloproteinases (TIMPs), that inhibit metalloproteinase activity, and by upregulating plasminogen activator inhibitor 1 (PAI-1) which inhibits urokinase-type plasminogen activator (uPA) activity (Graham 1997). Conversely, antisense oligonucleotide-mediated inhibition of TGFβ-3 expression in explant cultures promoted trophoblast outgrowth (Caniggia et al. 1999). Contrary to TGFβ, activin promoted outgrowth and differentiation of villous explant cultures which was accompanied by expression of the EVT markers HLA-G and MMP-2 (Caniggia et al. 1997).

Besides classical growth factors, many different cytokines and chemokines are secreted from EVTs and decidual cell types, predominantly from macrophages and fibroblasts (Table 1.3) (Jokhi et al. 1997; Hanna et al. 2006; Hannan and Salamonsen 2007). Many of their respective receptors were identified on EVTs (Drake et al. 2004). Chemokines such as chemokine (C-X3-C motif) ligand 1 (CX3CL1), chemokine (C-C motif) ligand 14 (CCL14), chemokine (C-C motif) ligand 4 (CCL4) (Hannan and Salamonsen 2007), chemokine (C-X3-C motif) ligand 16 (CXCL16) (Huang et al. 2006) or chemokine (C-C motif) ligand 21 (CCL21) (Red-Horse et al. 2001), increased trophoblast migration or invasion *in vitro*. Similarly, the interleukins IL-1, IL-6, IL-8 or IL-11 secreted from uNK cells were shown to promote gelatinase activity and/or trophoblast invasion (Librach et al. 1994; Meisser et al. 1999; Paiva et al. 2007). Moreover, regulatory binding proteins such as IGF-BPs (Irving and Lala 1995; Chakraborty et al 2003), soluble TNF receptors (Knofler et al 1998b), soluble fms-related tyrosine kinase 1-VEGF receptor (sFlt-1) (Clark et al. 1998) a

Table 1.3 Cytokines that regulate trophoblast migration and/or invasion.

EVT, extravillous trophoblast; uNK, uterine natural killer; "+", stimulates,; "-", inhibits

CYTOKINE	CELL	EFFECT	REFERENCE
CX3CL1	macrophages, fibroblasts	+	Hannan and Salamonsen 2007
CCL14	macrophages, decidua, EVT	+	Hannan and Salamonsen 2007
CCL4	decidua, macrophages	+	Hannan and Salamonsen 2007
CXCL16	macrophages	+	Huang et al 2006
CCL21	macrophages	+	Red-Horse et al 2001
TNFα	decidua, macrophages	-	Bauer et al 2004
IFNγ	uNK cells	-	Lash et al 2006
IL-1	uNK cells	+	Librach et al 1994
IL-6	uNK cells	+	Meisser et al 1999
IL-8	uNK cells	+	Paiva et al 2007
IL-11	uNK cells	+	Paiva et al 2007

VEGF/PlGF antagonist, or soluble endoglin (sEng), a secreted TGFβ co-receptor (Venkatesha et al. 2006), have been identified at the fetal-maternal interface adding further complexity to the growth factor network regulating trophoblast invasion. Physiological levels of soluble fms-related tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) appear to be critical for normal progression of pregnancy since serum levels of these factors are elevated in patients with preeclampsia (Tjoa et al. 2007).

1.1.5.3 Interaction with the Extracellular Matrix (ECM)

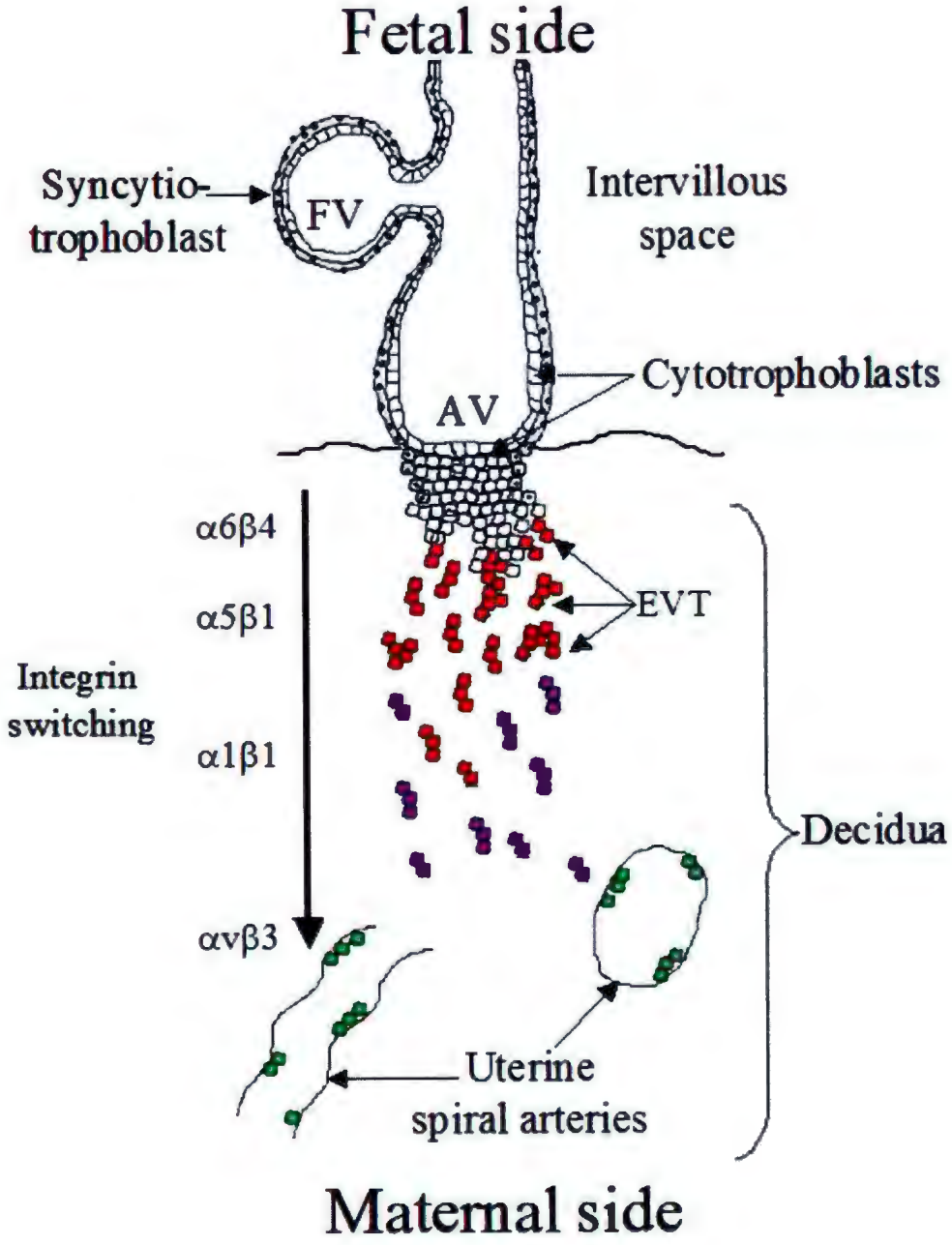
In order to properly invade the uterus a subpopulation of trophoblasts differentiates from epithelial-like cells to invasive cells (Kaufmann and Castellucci 1997). Multiple growth factors regulate trophoblast differentiation but this regulation is coordinated with the effect of the ECM that also interacts with the cells and controls the main cellular processes such as proliferation, survival, migration and invasion. The major cell surface receptors that cells use to assemble and recognize a functional ECM are integrins. Integrins are heterodimeric transmembrane proteins composed of α and β subunits (Hynes 2002). Eighteen α subunits and eight β subunits can assemble in 24 different combinations (Hynes 2002; Legate et al. 2009). The type of integrins expressed by a cell determines the ECM components that the cell can bind. It is widely recognized that activation of integrins is achieved by separating the α and β cytoplasmic tails as a consequence of talin binding to β-integrin membrane proximal sequences facilitated by kindlins (Wegener 2007; Wegener and Campbell 2008). Many proteins bind directly to integrins, yet only talin

and kindlins can regulate integrin affinity (Moser et al. 2009). Integrin activation increases the affinity of individual integrins for ECM ligands, but for a cell to bind strongly to ECM an increased avidity of the interaction is required by clustering integrins so that hundreds, or thousands, of weak interactions sum into a tightly bound adhesive unit. Ligand-bound integrins then recruit adaptor protein and kinases forming structures called nascent adhesions (Choi et al. 2008). A subset of nascent adhesions progresses to more stable dot-like focal complexes, which can mature into larger focal adhesions (FAs) and finally into streak-like fibrillar adhesions (Geiger 2001). Once integrins are activated and clustered they are able to transmit the vast array of intracellular changes collectively referred to as ''outside-in'' signalling (Legate and Fassler 2009). As integrin tails have no catalytic activity of their own, they must bind accessory molecules that help in cytoskeletal organization. Cells, therefore need to integrate the information coming from separate signals such as the ECM or many different growth factors, cytokines and hormones, and respond deciding whether to live or die, to divide or not, to migrate or invade, among other possibilities. One strategy that cells use to achieve specificity involves scaffold proteins which organize groups of interacting signalling proteins, both temporally and spatially, into signalling complexes. Database mining combined with an extensive literature search recently identified 156 signalling, structural, and adaptor molecules that comprise the ''integrin adhesome'' (Zaidel-Bar 2007; Legate et al. 2009). FA proteins involved in establishing and maintaining the integrin–cytoskeleton linkage can roughly be divided into four classes: (1) integrin-bound proteins that directly bind actin, such as talin (Jiang et al. 2003), α-actinin (Brown et al. 2006), and filamin

(Samuelsson et al. 2011); (2) integrin bound proteins that indirectly associate with and regulate the cytoskeleton such as kindlin, integrin-linked kinase (ILK) (Hannigan et al. 1996), paxillin (Laukaitis et al. 2001), and FAK (Schaller et al. 1995); (3) non-integrin-bound actin binding proteins, such as vinculin (Ziegler et al. 2008; Critchley and Gingras 2008); and (4) adaptor and signalling molecules that regulate the interactions of the proteins from the above-mentioned groups, such as actin-related proteins (Arp) 2/3, Wiskott-Aldrich syndrome protein (WASP) (Kiselar et al. 2007), and diaphanous-related formin (DRF) (Riveline et al. 2001) that control actin polymerization (Butler et al. 2006) and signalling molecules like Rho GTPases which control the spatial and temporal regulation of the actin assembly (Jaffe and Hall 2005). The most important GTPases for the regulation of actin dynamics at the FA are Ras-related C3 botulinum toxin substrate 1 (Rac1), Cell Division Cycle protein 42 homolog (Cdc42) and Ras homolog gene family, member A (RhoA) (Jaffe and Hall 2005, Legate and Fassler 2009). Experiments utilizing novel imaging technologies such as total internal reflection fluorescence (TIRF) microscopy and fluorescent speckle microscopy, in combination with structural, biochemical, and *in vivo* data, point to talin, vinculin, α-actinin, and ILK as the crucial structural elements of the integrin–actin linkage, as well as the main components regulating FA growth (Legate and Fassler 2009).

During human placental development the EVT cells undergo a "switch" in expression of adhesion molecules, particularly integrin receptor proteins which bind to specific ECM ligands, as they differentiate (Aplin 1993; Damsky et al. 1994; Vicovac and Aplin 1996) (Fig 1.3). Proximal cell columns of EVT cells, in the tip of

Figure 1.3 Integrin switching. Trophoblast cells switch the expression of integrins on their surface as they differentiate. This schematic drawing highlights, in different colours, the expression of different integrin receptors along the extravillous differentiation pathway: uncoloured represents $\alpha6\beta4$, red represents $\alpha5\beta1$, purple represents $\alpha1\beta1$, and green represents $\alpha v\beta3$ positive EVT. FV, floating villi; AV, anchoring villi; EVT, Extravillous trophoblast.


Fetal side
Syncytio-
trophoblast
FV
Intervillous
space
Cytotrophoblasts
AV
α6β4
α5β1
Integrin
switching
α1β1
αvβ3
EVT
Decidua
Uterine
spiral arteries
Maternal side

the anchoring villi are proliferative, rounded, cohesive cells marked by the high expression of α6β4 integrin (Damsky et al. 1992). More distally from the base of the column, the interstitial EVT cells have differentiated into isolated, highly invasive, pleomorphic cells that highly express α5β1 and α1β1 integrins which are receptors for fibronectin and type IV collagen (Kam et al 1999; Damsky and Ilic 2002). The importance of the integrin-ECM interaction to trophoblast differentiation is stressed by the fact that patients with preeclampsia show abnormal integrin expression at the surface of EVT cells (Zhou et al. 1993). It is also possible to recapitulate trophoblast differentiation *in vitro* as well as the integrin switching by exposing trophoblasts to collagen or Matrigel (Aplin 1993; Damsky et al. 1994; Vicovac et al. 1995). These results suggest that the adoption of an invasive trophoblast phenotype is mediated by integrin-regulated signalling.

1.2. Integrin-Linked Kinase

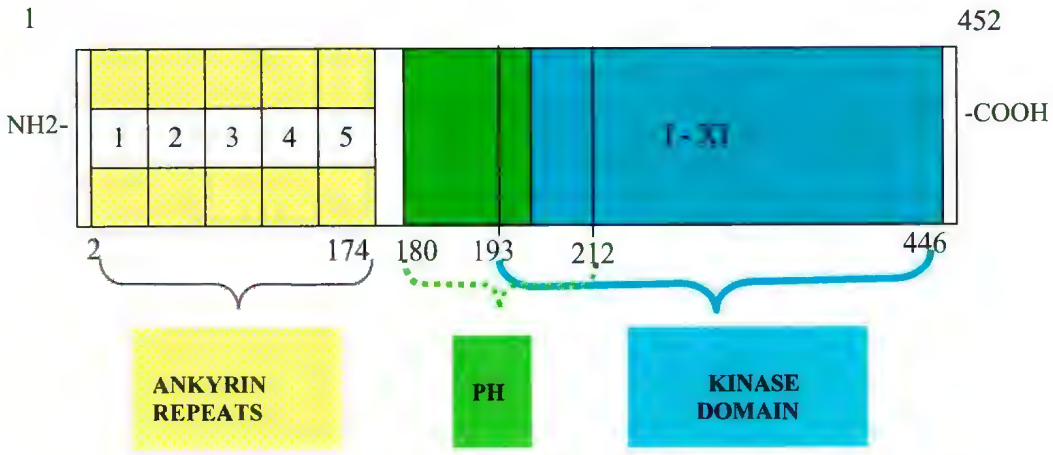
1.2.1 ILK Structure

ILK is a 55 kDa cytoplasmic protein recently described as a serine/threonine pseudokinase due to the unusual conformation of the kinase domain (Wickstrom et al. 2010). A detailed explanation of the kinase domain is included in Section 1.2.1.1. Ubiquitously expressed in mammalian tissues (Hannigan et al. 1996; Su et al. 2004), the gene encoding ILK is localized to human chromosome 11p15.5-11p15.4

(Hannigan and Dedhar 1997). A single isoform has been identified to date in all studied species (Legate et al. 2006). The protein (accession number Q13418, Uniprot database) contains 452 amino acids with three distinctive structural and functional domains: the kinase domain, the pleckstrin-homology (PH) domain and the ankyrin (ANK) domain (Fig 1.4).

1.2.1.1 The Kinase Domain: The C-terminal region of the protein contains a readily identifiable protein kinase catalytic domain divided into XI subdomains spanning from amino acids 193 to 446 (Melchior et al. 2002). Although there are a number of amino acids missing that are typically highly conserved in Ser/Thr protein kinases, ILK subdomains align with other representative protein kinases such as protein kinase A (PKA) and insulin receptor kinase (IRK) (Dedhar et al. 1999). Also the invariant lysine residue in subdomain II that is required for ATP binding is present in the ILK amino acid sequence. The GXGXXG sequence found in subdomain I in most kinases is replaced in ILK by the sequence NENHSG which puts ILK in the category of atypical kinases; however, there are other active protein kinases that lack all three glycine residues in the GXGXXG consensus like mitosis inhibitor kinase 1 (Mik1) and vacuolar protein sorting 15p (Vps15p) (Hanks et al. 1988). Mutational studies of catalytic domains of different proteins have confirmed that three highly conserved amino acid triplets, Histidine-Arginine-Aspartic acid (HRD), Aspartic acid-Phenylalanine-Glycine (DFG) and Alanine-Proline-Glutamic acid (APE) in subdomains VI, VII and VIII respectively, are crucial for kinase activity (Hanks et al. 1988). Of these three, only APE is present in ILK (Hannigan et

Figure 1.4 ILK protein domains. ILK contains a N-terminal ankyrin domain (yellow), a pleckstrin homology domain (PH) (green) and a C-terminal kinase domain (blue). The 5 ankyrin repeats are shown with numbers (1-5) and the 11 subdomains of the kinase domain are shown with roman numerals (I-XI).


1
452
NH2-
1
2
3
4
5
-COOH
2
174
180
193
212
446
ANKYRIN REPEATS
PH
KINASE DOMAIN

al. 1996; Hannigan et al. 2005). In fact, many mammalian kinases lack some or even the three conserved residues and are categorized as atypical kinases or pseudokinases. Some examples are members of the PI3K like family, ataxia-telangiectasia mutated protein (ATM), ATM and Rad3-related protein (ATR) and DNA-dependent protein kinase that lack the APE and have the HRD motif modified to Aspartic acid-Arginine-Histidine (DRH) (Walker et al. 1999). Importantly, mutations that replace the invariant glutamic acid (E) for a lysine (K) in position 359 of the APE sequence result in an inactive form of ILK (ILK-E359K) that functions as a dominant-negative (Delcommenne et al. 1998; Dedhar et al. 1999) impairing the binding to parvins. Altogether these comparisons confirm that ILK does not present a typical kinase domain it still has similar structure to other functional and important kinases (Boudeau et al. 2006). It is important to notice that the catalytic domain of ILK contains an integrin binding region that binds to β1 and β3 tails (Hannigan et al. 1996) and also mediates interactions of ILK with the actin binding adaptor proteins α-parvin, β-parvin and paxillin (Nikolopoulos and Turner 2000; Yamaji et al. 2001; Nikolopoulos and Turner 2001; Zhang et al. 2004; Chiswell et al. 2008; McDonald et al. 2008). These interactions are fundamental for the normal function of ILK as a regulator of cytoskeletal organization (Stanchi et al. 2009).

The unusual conformation of the kinase domain led to recent studies that concluded that ILK might be a pseudokinase (Wickstrom et al. 2010) and that it might regulate the phosphorylation of different substrates by acting as an adaptor protein, either to recruit a distinct S473 kinase activity or to inhibit a S473 specific phosphatase activity (Lynch et al. 1999). Knock-in mice carrying mutations in the

putative PH domain (R211A) or in the autophosphorylation site (S343A or S343D) are completely normal and do not show changes in AKT or glycogen synthase kinase 3 beta (GSK3β) phosphorylation or actin organization downstream of integrins (Lange et al. 2009). In accordance with this statement, Fukuda et al. (2011) studied the structure of the ILK kinase domain bound to its cytoskeletal regulator, the C-terminal calponin homology domain of α-parvin with crystallography. While maintaining a canonical kinase fold, the ILK kinase domain displayed a pseudoactive site conformation. They suggested that rather than performing the kinase function, this conformation specifically recognizes α-parvin to promote effective assembly of ILK into FA. The α-parvin-bound ILK kinase domain can simultaneously engage β1 and β3 integrin cytoplasmic tails. They also found that ILK is able to bind ATP but under their experimental conditions ILK was unable to hydrolyze the bound ATP. These results define ILK as a distinct pseudokinase that mechanically couples integrins and α-parvin to mediate cell adhesion (Fukuda et al. 2009). There are still controversies as it could be that α-parvin is inhibiting the ATP hydrolysis (Hannigan et al. 2011). Determination of the mechanisms and regulation of enzyme catalysis by the ILK kinase domain has not been completed yet due to the poor solubility and stability of ILK in solution so crystallography of pure protein has not been possible. Recently Maydan et al. (2010) was able to express full length-ILK in insect and mammalian cells and this should provide tools to better understand the biochemistry of ILK reactions (Maydan et al. 2010; Hannigan et al. 2011).

1.2.1.2 The Pleckstrin Homology Domain: The putative PH domain is located between amino acids 180 and 212. This motif is involved in the binding of

phosphatidylinositol phosphates. Phosphatydylinositol (3,4,5)-triphosphate (PIP3) is the secondary messenger product of PI3K and stimulates the kinase activity of purified recombinant ILK (Maydan et al. 2010; Boppart et al. 2011). Interestingly, integrin and growth factor-dependent stimulation of ILK activity is inhibited by inhibitors of PI3K and expression of constitutively active forms of PI3K also results in constitutively increased ILK activity, suggesting that ILK is a PIP3-dependent kinase (Dedhar et al. 1999). In order to study the role of the PIP3 binding motif in the regulation of PKB/AKT phosphorylation, Persad et al. (2001) mutated arginine 211 (R211) within the PIP3 binding motif. R211 is in the WKGRW motif (Tryptophan-Lysine-Glycine-Arginine-Tryptophan) that is important in binding phosphoinositide phospholipids in PH domains (Toker and Cantley 1997). When the PH domain of ILK was mutated in DU145 prostate cancer cells, AKT phosphorylation was reduced significantly and was not stimulated by addition of serum. These results strongly suggested that the PH domain is important for the activation of ILK, indicated in this case by the phosphorylation of AKT (Persad et al. 2001).

1.2.1.3 The Ankyrin Repeat Domain: the ANK repeat is one of the most common protein-protein interaction motifs in nature (Mosavi et al. 2002). *In silico* studies predicted four ANK repeats in the N-terminus but recently a crystallography study revealed a fifth ANK repeat when ILK is folded (Chiswell et al. 2010). The ILK consensus ANK repeat motif is a 33-amino acid repeat that folds into a hairpin-helix-turn-helix structure. Two anti-parallel helices from each repeat pack against one other, and the hairpin that links each pair of helices is oriented perpendicular to the helices to form a characteristic cross-sectional ''L'' shape (Sedgwick and Smerdon

1999; Mosavi et al. 2002). Within ANK repeat domains, adjacent repeats stack on top of one another such that the helices form helical bundles. The stacked repeats form a curved left-handed superhelical spiral. The interior surface of the spiral is concave and forms the ''ankyrin groove'', a feature that has been described as a cupped hand, with the helices as the palm and the hairpins as fingers. This concave surface provides an ideal recognition site for intermolecular interactions (Chiswell et al. 2008). Experiments designed to identify potential interactors of the ILK ANK repeat domain have identified the Linl-1, Isl-1 and Mec-3 (LIM)-domain protein (Way and Chalfie 1988) named Particularly Interesting New Cysteine-Histidine-rich protein (PINCH) as a candidate (Tu et al. 1999). In addition to binding ILK through LIM1, PINCH also binds indirectly to activated EGF receptor and PDGF receptor via non-catalytic region of tyrosine kinase adaptor protein 2 (Nck–2) forming a signalling complex coupling integrins and growth factors. This indirect connection between integrins, ILK and growth factor receptors is important for the coordination of the different signalling pathways regulated by the outside-inside signals (Dedhar et al. 1999). ILK ANK domain also binds to integrin-linked kinase-associated serine/threonine phosphatase 2C (ILK-AP) an ILK specific inhibitor (Leung-Hagesteijn et al. 2001; Kumar et al. 2004).

1.2.1.4 Other ILK Binding Partners: In a recent study, the Dedhar group identified new proteins associated with ILK utilizing "stable isotope labeling with amino acids in cell culture" (SILAC) and mass spectrometry techniques. Some examples of the newly described ILK-associated proteins include rapamycin-insensitive companion of mTOR (Rictor), α and β tubulin, RuvB-like1 and 2, HS1-

associating protein (HAX-1), T-complex protein 1 subunit and Ras-GTPase-activating-like protein 1 (IQ-GAP1) (Dobreva et al. 2008). Detailed analysis with immunoprecipitation, western blotting and colocalization analyses showed that when ILK is found in a complex with RuvB-1 and α-tubulin, α-parvin and PINCH are not present, suggesting that ILK forms distinct protein complexes throughout the cell (Dobreva et al. 2008). On a different set of studies, ILK, PINCH and parvins formed a ternary complex termed tIPP that localizes to FAs. These studies showed that their interaction is essential for the recruitment of this complex to the plasma membrane in response to outside-inside signalling and for coordination of cytoskeletal organization (Boulter and Van Obberghen-Schilling 2006; Legate et al. 2006; Stanchi et al. 2009). It also suggests that ILK function is likely regulated by interactions with different proteins in response to the need of the cell.

1.2.2 ILK Functions

ILK is an atypical serine/threonine kinase that binds to integrins directly and to tyrosine kinase receptors indirectly to coordinate outside-inside signals that control cell survival, proliferation and differentiation (Hannigan et al. 1996; Wu and Dedhar 2001). Several laboratories have demonstrated that ILK has direct targets for its kinase activities, phosphorylating AKT, GSK3β, myosin light chain and β-parvin (Lynch et al. 1999; Zhang et al. 2004; Legate et al. 2006; Boulter et al. 2006). There is still a lot of controversy and apparently the results from these communications might only be the consequence of partially purified ILK, as Fukuda et al. (2011)

working with highly purified recombinant ILK protein were unable to repeat the former results. In addition to its function as a kinase, ILK appears to be a key mediator of protein-protein interactions, an area where there is more consensus (Hannigan et al 2005; Legate et al. 2006; Fukuda et al. 2009). Deletion of ILK in C. elegans, Drosophila, or Danio (zebrafish) leads to muscle disconnection from the body wall due to the detachment of actin from the plasma membrane (Zervas et al 2001; Mackinnon et al. 2002; Postel et al 2008; Legate and Fassler 2009). In ILK-deficient mice, muscle detachment occurs at the level of the integrin–ECM interaction (Wang et al. 2008) suggesting that ILK regulates adhesion strength by reinforcing the integrin–actin connection and thus stabilizing the clustered state of integrins (Legate and Fassler 2009). Furthermore, ILK can undergo phosphorylation-dependent shuttling to the nucleus (Acconcia et al. 2007) where it appears to be important for maintenance of nuclear integrity, suggesting that ILK can be involved in a wide range of signalling pathways. In a recent publication, Butler et al. (2009) showed that ILK also plays an important role to promote syncytialization and hormonal differentiation of human trophoblast-derived BeWo cells, showing that ILK is able to coordinate signalling pathways that lead to differentiation of trophoblast cells.

Loss-of-function studies have stressed the importance of ILK in development and normal physiology. Embryonic lethality was observed in Xenopus laevis (Yasunaga et al. 2005) and in mice (Sakai et al. 2003) when ILK knockouts were developed. The lethality of the knockout model has made the delineation of ILK tissue-specific functions challenging. Fortunately, Cre-lox and RNA interference technologies have led to the design of different experimental strategies that have

allowed the study of the different tissue-specific roles of ILK (McDonald et al. 2008). The different studies with conditional knockouts have led to the conclusion that ILK is a multifunctional protein that can regulate several key cellular processes. ILK functions in bone, skin and central nervous system are related to cell adhesion, proliferation and migration (Terpstra et al. 2003; Belvindrah et al. 2006; Lorenz et al. 2007) while cells of the immune system rely on the adaptor and kinase capacities of ILK to maintain cell survival and normal tissue function. The regulation of each function seems to be tissue-specific as the mutation of ILK in chondrocytes (Terpstra et al. 2003), central nervous system (Graus-Porta et al. 2001; Belvindrah et al. 2006) and endothelial cells (Vouret-Craviari et al. 2004) reduces proliferation while in keratinocytes the conditional knockdown of ILK has the opposite effect and proliferation is increased (Lorenz et al. 2007). To date, only one study was conducted on the importance of ILK in trophoblast differentiation (Butler et al 2009).

1.2.3 ILK Signalling Pathways

As discussed previously, ILK is able to bind directly to integrins and indirectly to growth factors receptors creating a coordinating dock for several signalling pathways that control essential functions in living cells. The kinase activity of ILK is stimulated by integrins binding to extracellular proteins and soluble mediators including growth factors and chemokines (Delcommenne et al. 1998; Li et al. 2003; Xie et al. 2004; Rosano et al. 2006) in a PI3K-dependent manner (Delcommenne et al. 1998) while it is antagonized by phosphatases such as ILK-AP

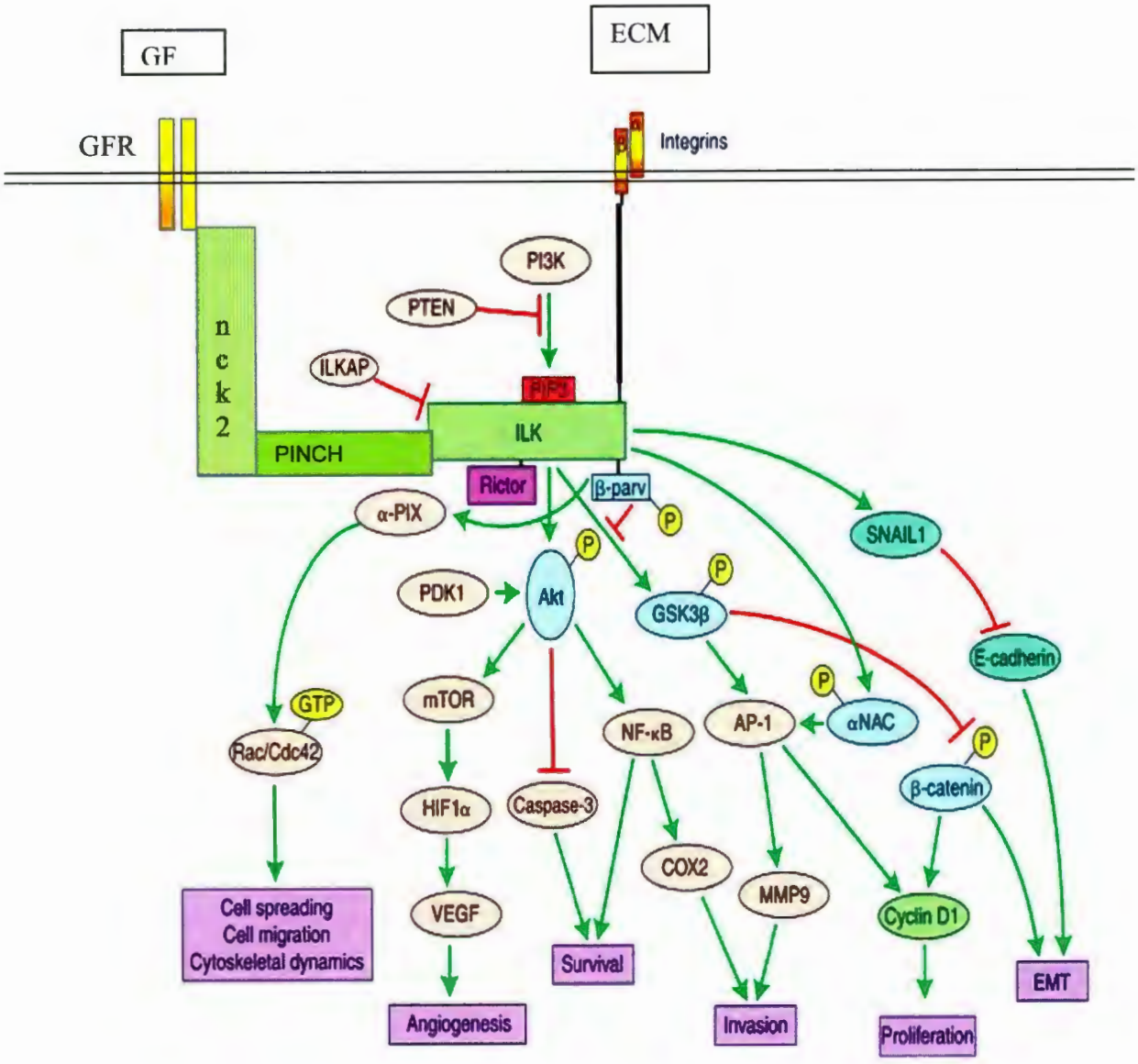
(Leung-Hagesteijn et al. 2001) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Persad et al. 2001). Following activation, ILK exerts control over a diverse set of downstream effectors that modulate crucial cellular functions. In particular, ILK regulates the phosphorylation of AKT at Ser 473 and of GSK3β at Ser 9 in various cell types (Delcommenne et al. 1998). By promoting phosphorylation of AKT, ILK stimulates signalling pathways that regulate cell survival, including those that involve the inhibition of caspase activation and the stimulation of nuclear factor (NF)-κB (Troussard et al. 1999; Persad et al. 2000; Hannigan et al. 2005; Acconcia et al. 2007). The phosphorylation of GSK3β by ILK regulates pathways that lead to the activation of activator protein 1 (AP-1) and β-catenin-LEF transcription factors, which in turn stimulate MMP9 and cyclin D1, respectively (Delcommenne et al. 1998; Novak and Dedhar 1999; Troussard et al. 2003) stimulating invasion or proliferation. ILK also phosphorylates nascent polypeptide-associated complex α (αNAC), a transcriptional coactivator of AP-1 (Quelo et al. 2004). Finally, ILK can modulate cell spreading, migration and cytoskeletal organization in mammary epithelial cells by activating p21 activated kinase (PAK)-interactive exchange factor α-PIX, a guanine-nucleotide exchange factor (GEF) for Rac1 and Cdc42 (Filipenko et al. 2005) and by activating cofilin through an interaction with phosphorylated Src as studied in rat intestinal epithelial cells (Kim et al. 2008). High levels of ILK expression reduced the expression of E-cadherin by stimulating the expression of its repressor, Snail through the stimulation of poly(ADP-ribose) polymerase 1 (PARP-1), thereby promoting epithelial-to-mesenchymal transition (EMT) and leading to invasion and metastasis in Spc2, a

mouse mammary epithelial cell line (McPhee et al. 2008). During placental formation, the stimulation of Snail expression in trophoblasts cells may be an integral component of ILK-induced downregulation of E-cadherin to facilitate the fusion of trophoblasts into syncytiotrophoblast (Butler et al. 2009). Fig 1.5 summarizes the most relevant ILK signalling pathways.

1.2.4 ILK in Cancer

ILK has been extensively studied in cancer cells where it appears to be upregulated in highly invasive tumors (Hannigan et al. 2005; Legate et al. 2006; Dillon et al. 2007). The expression of ILK is usually elevated in human malignancies and its expression is correlated with tumor stage and grade, for example, high levels of ILK expression correlates with poor patient survival in prostate cancer and melanoma (Graff et al. 2001; Dai et al. 2003). ILK expression is increased in human colon carcinomas, with greater expression in invasive regions of the tumors correlating with increased phosphorylation of GSK3β and nuclear translocation of β-catenin (Marotta et al. 2003; Hannigan et al. 2005). In patients with adenomatous polyposis ILK is consistently increased in the colonic polyps (Marotta et al. 2003), indicating that the alterations in ILK expression might regulate progression from benign disease to the different stages of cancer (Hannigan et al. 2005). It might also indicate that the lack of regulation of ILK could be a consequence of cell cycle control disruption. In ovarian cancers, ILK expression is increased relative to benign tumors and is not detectable in the normal ovarian surface epithelium. In this type of

Figure 1.5 ILK signalling pathways. Multiple signalling pathways are controlled by ILK directly or indirectly to regulate cell migration, angiogenesis, survival, invasion, proliferation and epithelial-mesenchymal transition (EMT). ECM, extracellular matrix; GF, growth factors; GFR, growth factor receptor; PI3K, Phosphoinositide 3 kinase; PTEN, Phosphatase and Tensin homolog deleted on Chromosome 10; PIP3, Phospatidylinositol 3,4,5-triphosphate; PINCH, Particularly Interesting New Cysteine-Histidine rich protein; βparv, β-parvin; αPIX, α-PAK-interactive exchange factor ; GSK3β, Glycogen synthase kinase β; AP-1, Activator protein 1; αNAC, α-Nascent polypeptide-Associated Complex; NF-κB, Nuclear Factor-κB; COX2, cyclooxygenase 2; ILK-AP, Integrin-Linked Kinase-Associated serine/threonine Phosphatase 2C; HIF-1α, Hypoxia Inducible Factor-1α; VEGF, Vascular Endothelial Growth Factor; Rictor , Rapamycin-insensitive companion of mTOR, PDK1, 3-phosphoinositide-dependent kinase 1; Nck-2, Non-catalytic region of tyrosine kinase adaptor protein-2; mTOR, Mammalian target of rapamycin; AKT, AKT8 virus oncogene cellular homolog; MMP9, matrix metalloproteinase 9; Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, Cell division cycle protein 42 GTP homolog (Adapted from McDonald et al. 2008 with permission).


GF
ECM
GFR
Integrins
nck2
PI3K
PTEN
ILKAP
PIP3
ILK
PINCH
Rictor
β-parv
α-PIX
SNAIL1
PDK1
Akt
GSK3β
E-cadherin
GTP
Rac/Cdc42
mTOR
NF-κB
AP-1
αNAC
β-catenin
HIF1α
Caspase-3
COX2
MMP9
Cyclin D1
VEGF
Cell spreading
Cell migration
Cytoskeletal dynamics
Survival
EMT
Angiogenesis
Invasion
Proliferation

cancer the levels increase with tumor progression (Ahmed et al. 2003). Interestingly this research group found that the serum levels of ILK are significantly increased in patients with invasive ovarian carcinomas postulating ILK as a novel marker for ovarian carcinoma (Ahmed et al. 2004).

Recent studies in thyroid tumors showed that ILK is strongly expressed in tumor tissue but not in normal thyroid tissue as evaluated with a tissue microarray assay (Younes et al. 2005). Very recently a study of patients with primary non-small-cell lung cancer (NSCLC) tumours showed that out of 138 tumours, 88 (64%) presented an ILK immunoreactivity, which varied significantly between various histological subtypes as it ranged from 46% positive in squamous cell carcinoma (SCC) to 79% positive in adenocarcinoma. The 5-year cancer-related survival and the recurrence-free survival (RFS) of ILK-positive SCC patients was significantly shorter than the patients with ILK-negative tumors (Watzka et al. 2011) postulating ILK serum level as a candidate marker for cancer progression.

1.2.5 ILK in Development

The development of multicellular organisms requires integrin-mediated interactions between cells and their extracellular surroundings (Montanez et al. 2008). Integrin binding to extracellular matrix regulates the assembly of multiprotein complexes, which transduce signals that regulate many aspects of cellular physiology such as survival, proliferation, shape, polarity, gene expression and differentiation (Hynes 2002; Lange et al. 2009). During the peri-implantation period of mammalian

development, the primitive endoderm forms on the surface of the inner cell mass (ICM) of the blastocyst and deposits a basement membrane (BM). The BM is required for the adjacent ICM cells to polarize and establish the columnar epiblast (primitive ectoderm) and for the remaining ICM cells to undergo apoptosis, resulting in the establishment of the proamniotic cavity (Coucouvanis and Martin 1995). In the absence of ILK, the primitive endoderm differentiates and produces a BM; however, there is a severe impediment to subsequent cavitation and polarization of ICM cells to form columnar epiblast cells (Sakai et al. 2003). This defect varies from the one observed in β1-integrin deficient mice, which die of an inability of the primitive endoderm to produce laminin-α1 and hence a BM (Fassler and Meyer 1995; Aumaillet et al. 2000; Li et al. 2002; Li et al. 2003). If one overcomes the defect by exogenous addition of laminin, the β1-null Embryoid Bodies can lay down a BM, differentiate and polarize an epiblast, and form cavities (Li et al. 2003). These data indicate that there are β1-integrin functions independent of ILK, but also is evidence that ILK functions independently of β1-integrin during the peri-implantation period. Mice lacking lLK failed to polarize epiblast cells and consequently, died during the peri-implantation period at embryonic day (E) 5.5 (Sakai et al. 2003). Embryonic lethality was also observed in ILK-deficient X. laevis (Yasunaga et al. 2005).

Studies of the expression of ILK during mouse development showed that ILK is highly expressed in the heart and somites at E 8.5 and E10.5 (Sakai et al. 2003) but the necessity of ILK for mouse trophoblast lineage development *per se* was not examined and remains unknown.

1.3 Abnormalities in Placental Development: Preeclampsia

Preeclampsia is a pregnancy-specific syndrome that affects 3–5 % of pregnant women worldwide (ACOG 2002; WHO 2005; Redman and Sargent 2011) and is one of the most frequently encountered medical complications of pregnancy (Young et al. 2010). Gestational hypertension in pregnant women is defined as 140 mmHg or higher and/or diastolic blood pressure of 90 mm Hg that occurs at 20 weeks of gestation or later in a woman with previously normal blood pressure (ACOG 2002). One quarter of the women with gestational hypertension develops proteinuria and that is the time when the condition is called preeclampsia (PE). PE may also be associated with edema, visual disturbance, headache and epigastric pain. Laboratory abnormalities may include hemolysis, elevated liver enzymes and low platelet count (HELLP Syndrome) which occurs in 20 % of women with severe PE (Creasy and Resnik 2000). Early onset PE refers to syndromes that start before week 34 of gestation while late onset refers to development after 34 weeks of gestation (von Dadelszen et al. 2003). If untreated, the syndrome can develop into seizures threatening the life of the mother and the fetus, a syndrome known as eclampsia (ACOG 2002; Young et al. 2010). The only available treatment at the moment for PE is the delivery of the baby at the time of diagnosis. For that particular reason PE is one of the most common reasons for induced preterm delivery. In developing countries it is still one of the leading causes of maternal mortality and morbidity. In Newfoundland a recent study showed that the PE incidence ranges from 4.2 % to 11 % depending on the geographical area. The same study showed that there is higher

risk in women with sisters that had preeclampsia suggesting that genetic and/or environmental factors may be affecting the incidence of the disease (Parfrey et al. 2002; Dawson et al. 2002).

Although there has been a wide range of studies on PE, the pathogenesis of the syndrome is still unknown. Normally by week 22 of gestation the remodelling of the maternal arterioles is complete rendering non-resistant vessels that allow sufficient blood to reach the placenta (Kaufmann et al. 2003). In PE invasive trophoblasts fail to fully remodel the maternal arterioles of the decidua and the inner third of the myometrium (Pijnenborg et al. 1982; Kaufmann et al. 2003) so the placenta shows a higher resistance to blood flow. It is well known that PE originates in the placenta and appears to progress in two stages: a preclinical one with poor placentation and, as a consequence, low placental blood supply. At this stage the patients do not show any signs or symptoms. There is a second clinical phase (Redman and Sargent 2011) when the patient develops the characteristic signs: hypertension and proteinuria. It is believed that the dysfunctional placenta during the preclinical stage releases factors into the maternal circulation that cause the clinical features of this condition. One of the first studies on the pathogenesis of PE associated the syndrome with abnormal expression of adhesion molecules by cytotrophoblasts (Zhou et al. 1993) and with altered regulation of TGFβ-3 (Caniggia et al. 1999). Interestingly, a recent study showed that the mechanisms of oxygen sensing were altered in PE as shown by lower levels of HIF1-α in explants exposed to hypoxia from patients with early onset of PE when compared with normal pregnancies (Rolfo et al. 2010). The production of placental anti-angiogenic factors,

specifically sFlt1, a soluble form of the VEGF receptor and sEng, are also upregulated in PE (Karumanchi and Stillman 2006; Yinon et al. 2008). Apparently these soluble factors compete for VEGF with the non-soluble VEGF receptor on the endothelial cells resulting in endothelial dysfunction. This group not only detected an increase in serum levels of these two solubles markers in PE patients but also were able to develop for the first time a rat model of preeclampsia by injecting adenovirus expressing sFlt1 through the tail vein of Sprague-Dawley rats at days 8–9 of pregnancy (Karumanchi and Stillman 2006). Although there has been an extensive array of studies on PE, the molecular basis for placental dysregulation of these pathogenic factors remain unknown so a better understanding of the mechanisms involved in the control of invasion may improve the prevention of PE or provide tools for an early diagnosis or a better treatment (ACOG 2002) .

1.4 Experimental Models used for the Study of Trophoblast Differentiation

1.4.1 Trophoblast Cell Lines

Several investigators have established trophoblast cell lines derived from first trimester human placentas which are commonly used to study trophoblast invasion. HTR8-SVneo cells were generated by transformation of HTR8 cells, obtained after plating first trimester human chorionic villi onto Matrigel, with the gene encoding simian virus 40 large T antigen and display an unlimited life-span in culture (Graham

et al. 1993). These cells share features with invasive trophoblasts such as expression of cytokeratin 18 and some EVT-specific integrins such as α5 integrin (ITGA5) (Graham et al. 1993). The cells are non-tumorigenic in nude mice and respond to TGFβ-3 growth inhibition in a dose dependent manner (Graham et al. 1993). However, one also has to be aware of the disadvantages of working with cell lines *in vitro*. The growth factors and cytokines that are produced by the surrounding cells in the decidua *in vivo* might be lacking in the *in vitro* model. So the behaviour of the studied trophoblast cell lines might not be reflecting the response to normal control mechanisms during pregnancy. Cell lines still prove to be useful because they are easy to manipulate and to study providing very valuable preliminary data before planning the experiments *in vivo* (Aplin et al. 2006).

Another useful model for *in vitro* studies of placental development is the villous explant culture. The system consists of culturing small pieces of chorionic villi on artificial ECM called Matrigel that allows the study of trophoblast column formation and EVT migration/invasion in a time- and distance-dependent manner (Genbacev et al 1993b). This system has the advantage that after plating villous tissue pieces on ECM-coated dishes, EVT develop and it is possible to study the expression of cell-specific markers such as HLA-G and integrins α5β1 and α1β1 (Vicovac et al. 1995; Bauer et al. 2004) under different experimental conditions. Explant cultures correctly mimic the EVT differentiation program (Miller et al. 2005), however, the different processes, i.e., adhesion, proliferation and migration/invasion, cannot be studied separately.

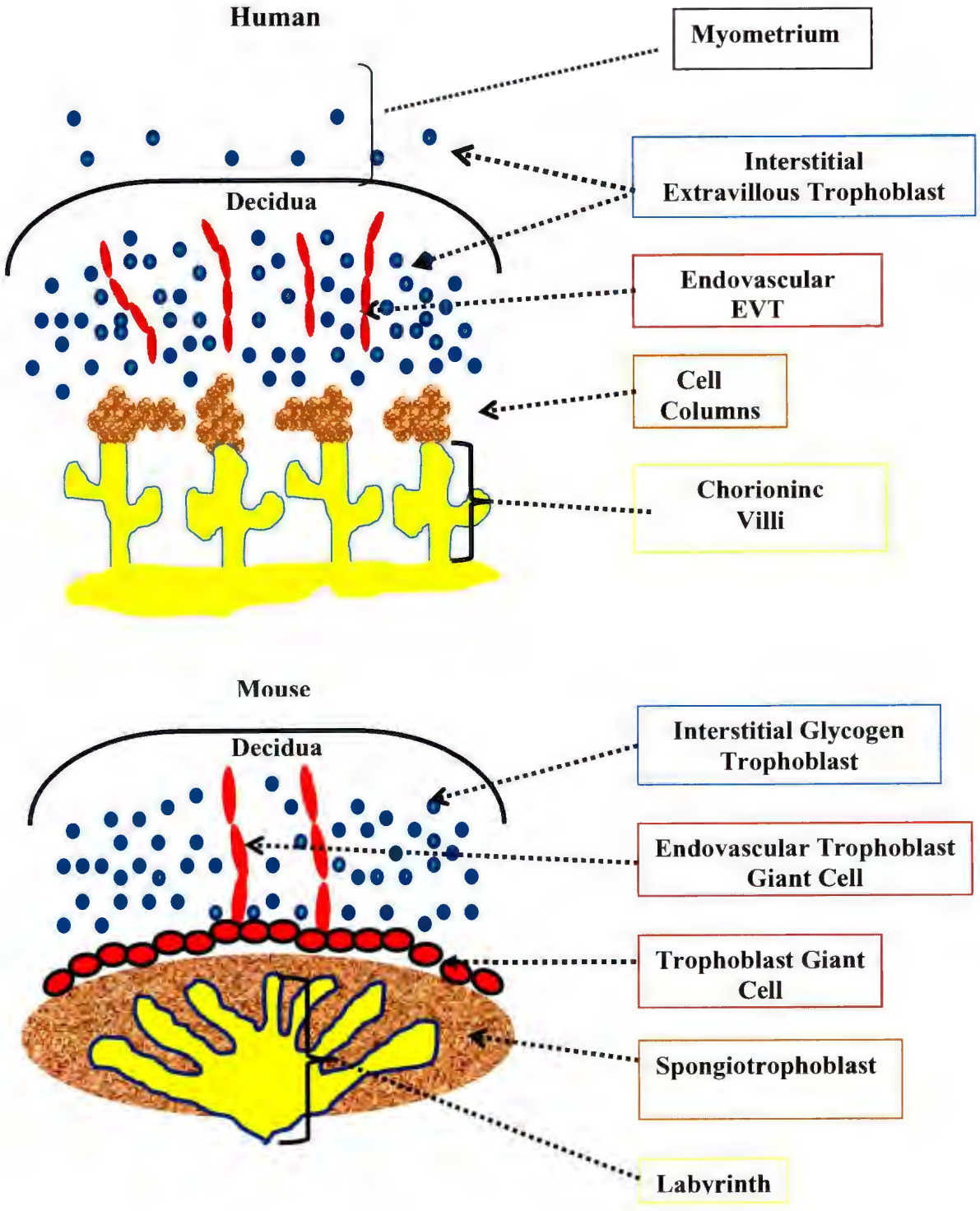
1.4.2 Mouse as a Model for Placentation

The mouse has become an important model system for studying the function of genes in mammals because of the ability to modify gene functions through transgenic and gene knockout approaches (Natale 2006). They also have the advantage of short reproductive times and large litters. Small mammals, compared to primates or the sheep model, also require less space making housing costs lower. A major benefit of mice compared to other rodents lies in the availability of embryonic stem cells which facilitates gene targeting and the development of transgenic lines (Carter 2007).

Structurally and functionally there are many valuable analogies between the mouse and human placenta. They are both chorio-allantoic as the tissue separating circulating maternal and fetal blood consists of chorionic trophoblasts, allantoic mesenchyme and vasculature (Georgiades et al. 2002). They are also both considered hemochorial because the trophoblasts are in direct contact with the maternal blood (Pijnenborg et al. 1981). Placentation in both mice and humans involves the development of three physiologically and anatomically distinct regions (Fig 1.6): 1) labyrinth, analogous to the fetal placenta in humans, 2) the junctional zone or spongiotrophoblast, analogous to the basal plate in the human placenta and 3) the decidua in mouse and the placental bed in humans, although they do not present many analogies they are both located at the boundaries of the placenta.

The labyrinth in the mouse can be considered analogous to the fetal placenta because this is where fetal and maternal blood circulates in close association for

Figure 1.6 Comparison of the human and mouse placenta main structures. These two diagrams show the analogous structures of the human and mouse placenta in the same colour. EVT, extravillous trophoblast. Note that the interstitial extravillous trophoblasts invade the myometrium in the human placenta but not in the mouse placenta (Adapted from Cross et al. 2003b).


Human
Myometrium
Interstitial Extravillous Trophoblast
Decidua
Endovascular EVT
Cell Columns
Chorioninc Villi
Mouse
Decidua
Interstitial Glycogen Trophoblast
Endovascular Trophoblast Giant Cell
Trophoblast Giant Cell
Spongiotrophoblast
Labyrinth

physiological exchange. The two structures have different branching patterns off the main projections of the chorionic plate. The human placenta maintains a tree-like structure with innumerable branches all having blunted-ended extremities. On the other hand, the branches off the main projections in the mouse are interconnected and form a maze like pattern (Georgiades et al. 2002). In mice, the ultrastructure has a slightly different organization than the human analogue. The trophoblastic portion of the mouse placenta consists of three layers. The layer lining the maternal blood spaces consists of a mononuclear trophoblast layer, in contrast with humans that have the syncytium in direct contact with maternal blood. Surrounding the mouse cytotrophoblast there are two layers of syncytiotrophoblast resting on a basement membrane shared with the fetal endothelia. Similar to human placental development, the cytotrophoblasts give rise to the syncytiotrophoblast by fusion, but the mouse cytotrophoblasts are the main hormone production source in the placenta unlike human cytotrophoblast (Benirschke and Kaufmann 2000; Georgiades et al. 2002). A recent study attempted to assess the molecular similarities or differences of mouse and human placenta by collecting protein and mRNA expression data through shot-gun proteomics and microarray expression analysis of this highly vascular exchange region, microdissected from the human and mouse near-term placenta (Watson and Cross 2005). Over 7000 orthologous genes were detected with 70% co-expressed in both species in the labyrinth and human fetal placenta suggesting that the analogies are also present at the genomic level. More than 80% of the genes known to cause placental phenotypes when disrupted in mice are expressed in human placenta

making these genes good candidates to study if they are involved in human placental pathologies, such as PE and IUGR (Watson and Cross 2005).

The mouse junctional zone, also called spongiotrophoblast layer, and the surrounding secondary trophoblast giant cells could be considered as the analogue of the basal plate (Georgiades et al. 2002). In both species this zone contains zygote-derived cytotrophoblasts but neither syncytiotrophoblast nor fetal blood vessels. The area is traversed by trophoblast-lined maternal arterial and venous channels. In mice there are two types of cytotrophoblasts present, spongiotrophoblasts and trophoblast glycogen cells (GlyC) which are derived from the spongiotrophoblasts (Coan et al. 2006). The Gly C are the only zygote-derived trophoblasts that express uPA, a secreted proteolytic enzyme involved in cell migration (Teesalu et al. 1998) that is also expressed in EVT. Also, Gly C are the only trophoblast cells to invade the decidua after E13 (Coan et al. 2006). The localization pattern and the fact that they give rise to the invasive cells of the placenta suggest that the spongiotrophoblasts can be considered comparable to the cell columns of the human placenta while the Gly C can be analogous to the interstitial EVT (Cross et al 2003 a). Interestingly, *Mash 2* is expressed in the spongiotrophoblasts and its human orthologue (MASH2 or HASH2) is detected in cytotrophoblasts cell columns (Alders et al. 1997; Janatpour et al. 1999; Hemberger and Cross 2001) and in both organisms this gene is downregulated during trophoblast differentiation into invasive trophoblasts, which are the Gly C in mouse and interstitial EVT in humans. The maternal arteries in the mouse decidua are lined by endovascular trophoblast giant cells (TGC) that can be considered the analogues of the endovascular EVT. The secondary endovascular TGC are large polyploid cells

that form through the process of endoreduplication. They are invasive and phagocytic and first mediate the invasion of the implanted conceptus into the maternal decidua (Zybina et al. 2000; Simmons et al. 2007). In addition they produce paracrine and endocrine factors including angiogenic factors (Lee et al. 1988; Carney et al. 1993; Voss et al. 2000), vasodilators (Yotsumoto et al. 1998; Gagioti et al. 2000), prolactin/placental lactogen 1 (Pl1/CSH1) (Wiemers et al. 2003), placental lactogen 2 (Pl2/CSH2) and proliferin (Plf) (Lee et al. 1988; Yamaguchi et al. 1992; Carney et al. 1993; Hamlin et al. 1994). It is important to note that the TGC are a heterogenous group of cells that are found lining the maternal blood spaces in the labyrinth, the maternal canal and the spiral arteries. The expression of genes from the prolactin and cathepsin families define subsets of TGC: those in the spiral arteries express Plf only, the ones lining the maternal canal are Plf and Pl2 positive while the ones present in the labyrinth express Pl2 and cathepsin (Simmons et al. 2007). The parietal TGC are easily identified by their location in the boundaries with the maternal decidua and their large nuclei that are significantly larger than the nuclei of all the other cells that form the mouse placenta (Simmons et al. 2007). At the molecular level, interstitial EVT cells, in humans, and parietal TGC in rodents, share expression of the matrix-degrading enzyme MMP9 (Librach et al. 1994) and the adhesion molecule $\alpha 1\beta 1$ integrin (Sutherland et al. 1993; Damsky et al. 1994).

The third layer is the maternal uterine tissue bordering the maternal side of the murine TGC zone which could be considered analogous to the human placental bed. This area becomes progressively invaded by zygote-derived trophoblasts in both species (Bischof et al. 2002). Trophoblast invasion in human placentas normally

extends up to, but not beyond, the inner third of the myometrium (Brosens et al. 1967; Pijnenborg et al. 1982). In contrast, in murine placentas, trophoblast invasion is evident throughout the decidua basalis but not the myometrium (Redline and Lu 1989). Thus, the murine decidua basalis and the human placental bed could be considered as analogous regions as far as their relative location but the extent of trophoblast invasion is different (Georgiades et al. 2002). Caution should still be taken when extrapolating conclusions based on analysis of the maternal structure in this layer as the mouse myometrial arteries are only slightly modified with loss of the smooth muscle layer but no replacement of the endothelial cell has been detected as in human myometrial arteries (Pijnenborg 1988; Georgiades et al. 2002; Adamson et al. 2002).

1.5 Rationale

Placental dysfunction is likely the major cause of two of the most common and serious complications of human pregnancy, maternal PE and IUGR (ACOG 2002; Redman and Sargent 2011). Each disorder affects around 5% of all pregnancies and is associated with serious morbidity and mortality, and the only known treatment to date is premature delivery, which places the baby at high risk of prematurity-related complications (Roberts and Gammill 2005; Mari and Hanif 2007). Even though PE and IUGR have been intensely studied for decades, the molecular pathways, etiology and pathogenesis of these diseases remain poorly understood (Cox et al. 2009). Despite the fact that cancer cells and trophoblasts share many common

characteristics (Lala et al. 2002; Ferretti et al. 2007), the invasion by trophoblasts is confined to specific areas of the pregnant uterus and little is known about the mechanism(s) that regulate the fine tuning of this process. MacPhee et al. (2001) discovered that FAK is involved in invasive trophoblast differentiation and described that it is only expressed until weeks 10-12 of gestation so there must be other FA proteins that are regulating the organization of FA and the differentiation of the trophoblast cells. One candidate is ILK because it is involved in many of the signalling pathways also known to control trophoblast differentiation, especially the outside-inside signalling pathway involving integrins and crosstalk between integrins and growth factor receptors (Legate and Fassler 2009). Thus, I hypothesize that ILK plays a crucial role in the differentiation of trophoblasts into invasive cells. This work will contribute to the overall understanding of trophoblast differentiation and help to elucidate some of the mechanisms that might lead to pregnancy-related pathologies.

1.6 Objectives

1) To determine the temporal and spatial expression of ILK in the human placenta: After written consent, human placentas from 1st and 2nd trimester elective terminations and deliveries at term were studied by immunofluorescence and immunoblot analysis to describe the temporal and spatial pattern of ILK expression in human placenta.

2) To study the role of ILK in trophoblast migration and invasion. Human placental explant derived trophoblasts named HTR8-SVneo were transiently transfected with different ILK constructs and used to study if ILK was necessary for migration and invasion of this cell line.

3) To determine the contribution of the oxygen environment on the regulation of ILK expression in the placenta. Human placental explants and HTR8-SVneo cells were exposed to different oxygen tensions in order to study the effect of oxygen in ILK expression and/or activity.

4) To compare ILK expression in PE vs normal placentas of human patients. Placentas were studied by immunofluorescence in an attempt to determine if the levels of ILK were altered in placentas from pregnancies complicated by preeclampsia in comparison to placentas obtained from normal pregnancies.

5) To analyze ILK expression in the mouse placenta and determine the feasibility of a trophoblast lineage specific knockout of ILK in mice. To date there are no data on ILK expression in mouse placenta and since the mouse is a good experimental model the spatiotemporal pattern of ILK expression was studied by immunofluorescence and immunohistochemistry.

CHAPTER 2

MATERIALS AND METHODS

2.1 Human Placenta Tissue Collection

Ethics approval for this study (protocol # 03.44) was obtained from the Human Investigation Committee of Memorial University of Newfoundland and the Health Care Corporation of St. John's Research Proposals Approval Committee. All study participants with confirmed ultrasound-dated pregnancy provided written consent.

Placental tissues were collected from first and second trimester elective terminations by the attending obstetrician utilizing dilatation and curettage. Samples were collected at the following weeks of gestation: 6-8 (n=16), 9-12 (n=27) representing the first trimester, 13-15 (n=6) for the second trimester and 37-42 (i.e., term; n=25). Placental tissues were collected in sterile phosphate buffered saline (PBS) and transported on ice to the laboratory within 10 min of the procedure. After washing and dissecting of tissue in cold PBS, samples were fixed in 4% paraformaldehyde (PFA) in PBS or Zinc-buffered fixative (ZBF) overnight with agitation or snap frozen in liquid nitrogen and stored at -80 °C.

Placental tissues from term pregnancies were collected at the case room (Janeway Children's Hospital, St. John's, NL) immediately after delivery. Term placentas were dissected and 3 pieces each from the central fetal side, peripheral fetal

side, central maternal side and peripheral maternal side were placed in cold PBS for extensive washing and subsequently fixed in either 4% PFA or ZBF or snap frozen in liquid nitrogen and stored at -80 °C.

2.2 Mouse Breeding and Mouse Placenta Collection

Experimental procedures were approved by the Animal Care Committee of Memorial University of Newfoundland (Protocols # 07-03-DM to 08-03-DM) and conducted in accordance with the guidelines established by the Canadian Council on Animal Care. C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and housed in microisolator cages with exposure to a 12 h dark and 12 h light cycle. Mice were mated and the day a vaginal plug was evident was designated as E0.5. Mice were anesthetised with CO_2 and euthanized by cervical dislocation. Mouse placentas were collected from pregnant mice between E7.5 and E17.5. Implantation sites, including the uterine wall, were collected in diethylpyrocarbonate (DEPC)-treated cold PBS and fixed in 4% PFA with rotation overnight at RT. The following day they were washed in RNase free PBS for 24 h and refrigerated until they were processed for paraffin embedding.

2.3 Tissue Processing and Paraffin Embedding

The mouse and human placental tissues were processed and paraffin embedded by the Histology Unit in the Faculty of Medicine at Memorial University of Newfoundland. Tissues were dehydrated in an ethanol series (70 %, 80 %, 2 X 95 %, 2 X 100 %) for 1 h at RT followed by xylene penetration (2 X 100% for 1 h) and paraffin infiltration (3 X 1 h) at 60 °C and a final paraffin infiltration for 1.5 h at the same temperature. Sections of paraffin-embedded tissues (5 μm thick) were prepared and utilized for immunofluorescence (IF) and immunohistochemistry (IHC). Some sections were stained with hematoxylin and eosin to examine tissue morphology. Periodic Acid-Schiff staining was also utilized to identify the GlyC in the mouse placenta (Natale 2006).

2.4 Immunofluorescence Analysis

For IF, following deparaffinization and rehydration, epitope retrieval was conducted by incubation of tissue sections with 0.1 % Trypsin/PBS for 10 min at RT followed by a wash in PBS. Sections were blocked in 5 % normal goat serum/1 % horse serum/1 % fetal bovine serum in PBS for 30 min at RT with gentle agitation. Samples were then incubated for 1 h at RT in primary antisera or affinity-purified IgG of the appropriate species at the same concentration as the primary antisera, to serve as a negative control. All the antisera used for experiments are listed in Table 2.1. After three washes in PBS, the tissue sections were incubated with appropriate Rhodamine-Red-X (RRX) or Fluorescein isothiocyanate (FITC)-conjugated secondary antisera. The sections were then washed with PBS containing 0.02 %

Table 2.1 Antisera utilized for immunofluorescence (IF) and immunohistochemistry (IHC) experiments. N/A, not applicable, the dilution depends on the primary antiserum used (see Chapter 2, Materials and Method, section 2.12)

Antisera	Method	Dilution	Supplier	Catalogue #
Mouse anti-ILK clone 65.1	IHC IF	1:200	Abcam, Ltd, Cambridge, MA, USA	AB49979
Rabbit anti-ITGA5	IF	1:250	Chemicon International, Temecula, CA	AB1928
Mouse anti-ITGAV	IF	1:100	BD Biosciences, Palo Alto, CA	611012
Mouse anti-pAKT1 (S 473), clone 587F11	IF	1:100	Cell Signaling Technology	4051
Mouse anti-Cytokeratin 7	IF	1:100	Chemicon International	MAB3554
Rabbit anti-VWF	IF	1:200	Chemicon International	AB7356
Rabbit anti-CSH1	IF	1:150	Chemicon International	AB954
Rabbit anti-Cx26	IF	1:150	Chemicon International	AB8143
Rabbit anti-Cx31/GJB3	IF	1:50	Abcam, Ltd, Cambridge, MA, USA	AB59925
Rabbit anti-laminin	IF	1:100	Abcam, Ltd, Cambridge, MA, USA	AB11575
Rabbit-anti-EGFP	IF	1:100	Clontech Laboratories, Inc	632377
FITC-Sheep anti-Rabbit IgG	IF	1:250	Sigma, St. Louis, MO, USA,	F7512
ChromPure Rabbit IgG	IF	N/A*	Jackson ImmunoResearch Labs, West Grove, USA	011-000-003
ChromPure Mouse IgG	IF	N/A*	Jackson ImmunoResearch Labs, West Grove, USA	015-000-003
HRP-Goat anti-Mouse IgG (H+L)	IHC	1:250	Promega Corporation, Madison, WI, USA	W402B
RRX-Donkey anti-Mouse IgG	IF	1:150	Jackson ImmunoResearch Labs Inc, West Grove, USA	715-295-150

Tween 20 (PBST) and mounted in Vectashield (Cat # H-1000; Vector Laboratories Inc., Burlington, Ontario, Canada). If double immunofluorescence experiments were used, incubations in additional primary and secondary antisera were employed as described above, following washes in PBST and blocking of tissue sections for 30 min. All slides were observed using a Leica DM-IRE2 inverted microscope (Leica Microsystems, Richmond Hill, Ontario, Canada) attached to a Retiga EXi CCD camera (QImaging, Burnaby, B.C, Canada) and equipped for epifluorescence illumination. Openlab Image Analysis software (Version 5.5; Improvision, Inc., Lexington, MA, USA.) was used for image capture and analysis. Analysis of mouse placenta was performed on at least three placentas from at least three different animals per gestational age collected.

2.5 Immunohistochemical Analysis

For IHC, after deparaffinization and rehydration, sections of mouse placenta were first subjected to antigen retrieval as described above and then treated with 3% H_2O_2 to block endogenous peroxidases. Following addition of primary antisera raised against ILK or IgG control and horseradish peroxidase (HRP)-conjugated secondary antisera (Table 2.1), colour development was conducted using the Vector Peroxidase Kit (Vector Laboratories, Inc, Cat # SK-4100). Tissue sections were then counterstained with Hematoxylin QS (Vector Laboratories Inc, Cat # H-3404), dehydrated and mounted in Permount (ThermoFisher Scientific).

2.6 Cell Culture

The HTR8-SVneo cell line was a kind gift from Dr. Charles Graham (Queen's University, Kingston, Ontario). This cell line, originally derived from primary human villous explants, has been extensively characterized and has become an accepted cell line model of EVT. It is invasive and non-tumorigenic and these cells have been used in the past for assessing trophoblast invasion (Graham et al. 1993; Leach et al. 2004; Kotani et al. 2009). Cells were maintained in RPMI 1640 media (Cat # 11875; Invitrogen Ltd) supplemented with 10 % fetal bovine serum (FBS) (Cat # CS-C08-500-U; Cansera International, Inc), 0.5 % Penicillin (100 U) and 0.5 % Streptomycin (100 μg) (Cat # 15140-122; Invitrogen). Cells were cultured at 37 °C in a humidified tissue culture incubator containing 5% CO_2 in air. For those experiments requiring serum starvation, cells were cultured in RPMI 1640 media with 0.5 % FBS according to Graham et al. (1993).

A low oxygen environment was created in some experimental settings to study the effect of oxygen tension on ILK expression and/or activity. For culture in low oxygen tension, the cells were placed in a C-chamber system attached to a ProOx 110 oxygen controller (Biospherix Ltd, USA) and connected to a 3% O_2/97 % N_2 tank and a probe that constantly monitored the level of O_2.

Hypoxic conditions were also mimicked by the addition of cobalt chloride ($CoCl_2$) to the culture media. Cobalt can substitute for ferrous iron and become incorporated into newly synthesized heme moieties. Unlike iron, cobalt binds O_2 with much lower affinity and thus locks the respective heme containing protein in a deoxy

conformation (Daghman et al. 1999). Desferrioxamine (DFX) was also added to the cell culture system to mimic hypoxia. Iron chelators such as desferrioxamine interfere with the binding of oxygen to ferrous heme also maintaining a deoxy status (Goldberg et al. 1988). In all experiments, the level of HIF-1α was assessed by immunoblot analysis to confirm that cells were indeed exposed to a low oxygen environment or in a hypoxia mimic situation.

2.7 Preparation of pEGFP Plasmids

2.7.1 Preparation of Plasmid DNA

To confirm the identities of plasmids that were obtained for experiments a small volume of each plasmid was cultivated and purified for subsequent restriction endonuclease digestion.

2.7.1.1 Mini Preparation of Plasmid DNA

The expression vectors pEGFP-C3 (enhanced green fluorescent protein), pEGFP-C3-ILK-WT, -E359K, -S343D and pcDNA3.1-ILK were obtained spotted onto Whatman paper from Dr. Greg Hannigan (Monash University, Australia). The spot on the paper was excised and eluted in 50 μl of Tris-EDTA (ethylendiaminetretraacetic acid) buffer (TE; 10mM Tris pH 8, 1mM EDTA) by incubating at 55 °C for 5 min, followed by centrifugation and storage at –20 °C until use.

DH5α *Escherichia coli* competent cells (Invitrogen, Library efficiency® DH5α™ Cat. #. 18263-012) were thawed on wet ice, gently mixed and aliquoted into 100 µl volumes in chilled polypropylene tubes. Approximately 1 µg of plasmid DNA was added to the competent cells and incubated on ice for 30 min followed by a heat-shock for 45 sec at 42 °C in a water bath and an incubation on ice for 2 min. YT media (0.9 ml consisting of 1.6 % Bacto tryptone, 1 % yeast extract, 0.5 % NaCl, pH 7) was added to the mixture and incubated for 1 h at 37 °C with agitation. Then 25 or 50 µl of the inoculated medium were each spread on agar culture plates containing ampicillin or kanamycin (depending on the plasmid) and incubated overnight at 37 °C. The following day, four sterile tubes of YT medium with ampicillin (50 µg/ml) or kanamycin (30 µg/ml) were each inoculated with one colony from the appropriate culture plates and incubated at 37 °C on a shaker overnight. Each 3 ml mini-culture was pelleted by centrifugation at 3000 rpm for 10 min at 4 °C, re-suspended in 100 µl of ice-cold glucose-TRIS-EDTA buffer (Solution I) and vortexed vigorously to remove any cell clumps and incubated for 5 min at RT. Two hundred microlitres of fresh 1 % SDS, 0.2 N NaOH (Solution II) was added to each mixture and mixed by inversion. The lysate was then incubated on ice for 5 min. One hundred fifty microlitres of 5 M potassium acetate (Solution III) was added to each tube, gently vortexed and incubated on ice for 10 min. The samples were then centrifuged at full speed for 15 min and the supernatant was transferred to a fresh tube and extracted with one volume of phenol/chloroform (1:1). After vortexing, the tubes were centrifuged for 5 min and the aqueous phase transferred to new tubes. One volume of

chloroform was added to each sample and the tubes vortexed and centrifuged for 5 min. The top aqueous phase was then transferred to new tubes.

The DNA was then precipitated with two volumes of ice-cold 95 % ethanol at -70 °C for 1 h. Following centrifugation at full speed for 5 min and discarding of the supernatant, the pellets were washed with two volumes of ice-cold 70 % ethanol, centrifuged and air dried. The DNA was resuspended in 50 µl of ultra pure water and quantified. Quantification of DNA was performed by UV absorbance spectroscopy (1 A260 O.D. Unit for dsDNA = 50 µg/mL). The plasmids were then stored at –20 °C.

2.7.1.2 Restriction Enzyme Assay

Every time a new vector was obtained and purified, the presence of the cDNA of interest was confirmed by restriction enzyme digestion assays. pEGFP-C3-ILK expression plasmids (pEGFP-C3-ILK-WT, -E359K, -S343D) were digested with HindIII (New England BioLabs; Cat # R0104S) and XhoI (New England Biolabs; Cat # R0146S) restriction enzymes while pcDNA3.1-ILK was digested with XhoI using a standard protocol. The following represented a 20 µl reaction mixture: 2 µl reaction buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 New England Biolabs Cat # B7002S), 2 ul of 1mg/ml bovine serum albumin (BSA), 1 µg DNA, 20 U each of Hind III and XhoI, and dH_20 to complete 20 µl. A control without enzyme and a control without plasmid DNA were included for each vector tested. The tubes were incubated for 1 h at 37 °C and after complete digestion of the

DNA, electrophoresis was conducted on 1 % agarose gels in 1X Tris-acetic acid-EDTA (TAE) buffer. Each digested DNA sample (20 µl) was mixed with 4 µl of 6X loading buffer and 15 µl of these prepared samples were loaded in each lane of the gel. In addition, 1 µg of 1 kb ladder (New England Biolabs, Cat # N3232L) was also run in a gel lane as a DNA standard marker. The gels were run at 80 V for 1 -1 ½ h. Pictures were taken with a KODAK Gel Logic 200 Imaging station (Mandel Scientific Company, Inc).

2.7.1.3 Midi Preparation of Plasmid DNA

After confirming the identity of the appropriate plasmids, 50 ml 2X YT media containing appropriate antibiotics were each inoculated with appropriate mini-cultures and grown overnight at 37 °C with agitation (midi-cultures). Plasmid DNA was subsequently isolated using a QIAGEN Midi-kit (Cat # 12143) according to the manufacturer's instructions.

Briefly, cultures were pelleted by centrifugation at 6000 g for 15 min at 4 °C, re-suspended in 6 ml of Buffer P1 and vortexed to remove any cell clumps. Six ml of buffer P2 were added; the mixture was shaken vigorously and incubated 5 min at RT to lyse the bacteria. Six ml of chilled buffer P3 was added to the lysate and mixed. Each lysate was poured into the barrel of a QIA filter cartridge and incubated at RT for 10 min. To separate contaminating proteins, genomic DNA and detergent from the plasmid DNA, the mixture was filtered through a QIA filter on to an affinity QIA

high speed mini column that had been previously equilibrated. After several steps of washing, the DNA was eluted from the column with 5 ml of QF Buffer and collected in a Falcon tube where the DNA was subsequently precipitated by adding 3.5 ml of RT isopropanol and incubating for 5 min at RT. The precipitated DNA was attached to a QIA precipitator. DNA was washed again with 70 % ethanol and dried before it was finally eluted with dH_2O.

Quantification was performed by UV absorbance spectroscopy (1 A260 O.D. Unit for dsDNA = 50 ug/mL). To assess DNA quality A260/A280 was measured and only plasmid DNA with ratios between 1.6 and 1.8 was used. All plasmids were stored at –20 °C.

2.8 Migration Assays

HTR8-SVneo cells were initially seeded on 22 × 22 cm glass coverslips (1×10^5 cells/coverslip) placed in 35 mm tissue culture dishes and cultivated in RPMI 1640 media, as described above, with the omission of antibiotics during transfection. When cells reached approximately 85% confluence, they were transiently transfected with empty pEGFP-C3 expression vector (BD Clontech, Cat # 6082–1), pEGFP-C3 containing human ILK-WT or pEGFP-C3 containing human ILK-E359K using Lipofectamine 2000 (Cat.#. 11668–027; Invitrogen Ltd.) according to the manufacturer's instructions. Transient transfections were conducted with 2 μg of DNA with a DNA:Lipofectamine ratio of 1:4. Before starting the definitive

experiments, different ratios of DNA: Lipofectamine were tested to determine the ideal ratio that yielded the most efficient transfection with the lowest volumes of lipofectamine.

Twenty-four hours after transfection, a wound was created in the confluent cell monolayers with a sterile pipette tip and cells were cultivated under serum starving conditions to study migration. The wounds were initially photographed (migration time=0 h) using a 10X objective under phase contrast and epifluorescence illumination with a DM-IRE2 inverted microscope (Leica Microsystems) equipped with a Retiga EXi CCD camera (QImaging). A second set of pictures was taken 24 h later (total of 48 h post-transfection) to assess cell migration into the wounds. The ratio of the number of transfected cells (i.e., EGFP-ILK fusion protein-containing cells) to the total number of cells in the wound after 24 h was subsequently determined from the captured images using Openlab Image Analysis software (Improvision). Migration assays were completed in triplicate and the experiment was repeated four times.

2.9 Invasion Assays

Following transient transfection of HTR8-SVneo cells with pEGFP-ILK expression vectors (as described in section 2.7), 5×10^4 cells were plated on growth factor-reduced Matrigel-coated transwell inserts containing 8 μm polyethylene terephthalate (PET) membranes (Millipore Inc.; Cat # PIEP12R48) and cultured under standard tissue culture conditions in RPMI 1640 media containing 0.5% BSA.

The lower chamber of the transwell contained RPMI 1640 media /10% FBS as an attractant. Under these culture conditions, HTR8-SVneo cells demonstrated an invasive phenotype characteristic of EVT cells (Graham et al. 1993, Kilburn et al. 2000). After 72 h of incubation, the transwells were washed with cold PBS and fixed with cold methanol for 3 min. The Matrigel was removed and each PET membrane was mounted on glass slides with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories; Cat #H-1200). The total numbers of transfected (EGFP-fusion protein-expressing) cells were counted on the underside of the membrane with a Leica DM-IRE2 inverted microscope (Leica Microsystems, Richmond Hill, Ontario, Canada) attached to a Retiga Exi CCD camera (QImaging, Burnaby, B.C, Canada) and equipped for epifluorescence illumination. Microscopic images were captured and analyzed with Openlab Image Analysis software (Version 5.5; Improvision, Inc., Lexington, MA, USA.). All invasion assays were conducted in triplicate and repeated three times.

2.10 MTT Assay

HTR8-SVneo cells were seeded (1×10^4 cells/well) in 96-well plates and cultured in RPMI 1640 media as described above. After transient transfection with the different pEGFP-C3 vectors, the cells were cultured under serum-starving conditions for 48 h and the assay was conducted following the manufacturer's detailed instructions (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide, Cell Proliferation Kit I, Cat. # 1 465 007; Roche Diagnostics, Laval, Quebec, Canada). Briefly, following 72 h of incubation, 10 µl of MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to each well of the plate for 4 h at 37 °C followed by the addition of a solubilisation solution and incubation overnight. The following day absorbance measurements (550 nm) were completed with a POLARstar Optima Plate Reader (BMG Laboratories, Offenburg, Germany). Assays were done in triplicate and the experiments repeated twice.

2.11 Immunoblot Analysis

Frozen tissue samples were pulverized under liquid nitrogen using a mortar and pestle and then homogenized in 500 µl of modified radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Triton X-100, 1 % sodium deoxycholate, and 0.1 % SDS) containing 100 µM Na_3VO_4 and Complete™ mini EDTA free protease inhibitors (Cat # 1 836 153, Roche Molecular Biochemicals)

For protein extraction of cell lines, cells were initially seeded at 2 x 10^5 cells/well in 6-well plates and cells were lysed by the addition of 500 µl/well of modified RIPA lysis buffer containing inhibitors. Cells were harvested with a plastic cell scraper and homogenized with a 27G needle attached to a 1 ml syringe. Samples were cleared by centrifugation and supernatants retained for protein assays and

immunoblot analysis. Sample protein concentrations were determined by Bradford Assay (Bradford 1976) (Cat # 500-0001, Bio-Rad Laboratories). Protein samples (40 μg/lane) and standards (Bio-Rad Cat # 161-0374) were separated in 10 % polyacrylamide gels under denaturing conditions (Laemmli 1970) and gels were electroblotted to 0.45 μm nitrocellulose membrane (Cat # 88018, Pierce). Blots were rinsed in Tris-buffer saline-Tween 20 (TBST; 20 mM Tris base, 137 mM NaCl, and 0.1 % Tween 20, pH 7.6) and blocked in either 5 % BSA/TBST or 5 % skim milk/TBST. Appropriate primary antibodies (listed in Table 2.2) at specific dilution factors, according to the manufacturer's suggestion, were incubated on blots either at 4 °C overnight or 1 h at RT, depending on the primary antibody, with constant agitation and then rinsed in TBST. Immunoblots were incubated in HRP-conjugated secondary antibody for 1 h at RT with constant agitation and then washed with TBST. The enhanced chemiluminescent reaction was developed with the Pierce Supersignal West Pico chemiluminescent substrate detection system (Cat # 34080, Pierce; Thermo Fisher Scientific) and multiple exposures were generated to ensure the linearity of the film response. Densitometric analysis of the bands, to normalize and compare bands of different gestational weeks or experimental treatments, were performed on scanned film images utilizing Image J software (NIH, USA).

2.12 Kinase Assays

HTR8-SVneo cells were seeded in 35 mm culture dishes at 2.5 x 10^5 cells/dish and

Table 2.2 Antisera utilized for immunoblot analysis

Antisera	Dilution	Supplier	Catalogue #
Rabbit anti-ILK	1:2000	Upstate, Waltham, MA	05-575
Rabbit anti-ILK	1:4000	Cell Signaling Technology, MA , USA	3862
Rabbit anti-AKT	1:1000	Cell Signaling Technology	9272
Rabbit anti-pAKT1 (Ser 473)	1:1000	Cell Signaling Technology	9271
Mouse anti-GSK3α/β	1:1000	Santa Cruz Biotechnology, CA, USA	sc-7291
Rabbit anti-pGSK3β	1:1000	Cell Signaling Technology	9336
Mouse anti-HIF-1α	1:250	BD Biosciences, Palo Alto, CA	610958
Mouse anti-ACTB	1:5000	Sigma, St Louis, MO, USA	A5316
HRP-Goat anti rabbit-IgG (H+L)	1:10000	Pierce Rockford, IL, USA	31460
HRP-Goat anti mouse IgG (H+L)	1:10000	Pierce Rockford, IL, USA	31430

cultured in either 20 % or 3 % oxygen conditions as described above for 24 h. Cells were lysed with Triton buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml Leupeptin containing Complete™ Mini EDTA-free protease inhibitors). Protein concentrations were determined by the Bradford method (Bradford 1976). For immunoprecipitation, 4.0 μg of mouse monoclonal ILK antisera (Upstate Cat # 05-575) or non-specific IgG (Jackson ImmunoResearch Labs Inc, Cat # 015-000-003) was added to 400 μg of appropriate HTR8-SVneo cell lysate and incubated overnight at 4 °C with gentle agitation. The following day, TrueBlot anti-mouse IP beads (50 μl) (Cat # 00-8811; eBiosciences, San Diego, CA, USA) were added to each sample and incubated for 2 h at 4 °C with gentle agitation. ILK antibody-bead complexes were centrifuged at 13,000 x g for 1 min at 4 °C and subsequently washed three times with 500 μl of Triton lysis buffer followed by three washes with 500μl of kinase buffer (Cat # 9802; Cell Signaling). Complexes were resuspended in 50 μl of kinase buffer supplemented with 1.0 μl of 10 mM ATP (Cat # 9804; Cell Signaling) and 1 μg of GSK3β fusion protein (Cat # 9237; Cell Signaling) and incubated for 30 min at 30 °C. The reaction was then terminated with 12 μl of 5 X sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) loading buffer followed by vortexing and centrifugation for 30 sec at 14,000 x g. All samples were heated to 95 °C for 5 min prior to loading on 10 % polyacrylamide gels according to Laemmli (1970) followed by electroblotting to 0.45 μm pore nitrocellulose membrane (Cat # 88018, Pierce; Thermo Fisher Scientific). Immunoblot analyses of phosphorylated GSK3β (Ser-9) (Cell Signaling Cat # 9336),

total GSK3 α/β (Santa Cruz Cat # 7291) and ILK proteins (Cell Signaling Cat # 3862) (See Table 2.2) were then conducted

.

2.13 Placental Explant Cultures

Isolation and culture of placental explants were conducted at the Samuel Lunenfeld Research Institute through a collaboration with the laboratory of Dr. Isabella Caniggia. Placentas from patients that underwent elective terminations, following informed consent, were collected in cold PBS, pH 7.4, by a registered nurse at the medical center. All the tissues were manipulated with smooth ended forceps and dissected with ophthalmologic scissors under a dissecting microscope.

The morning of chorionic villous dissections, Matrigel aliquots were taken from the –20 °C freezer and thawed at 4 °C. Prior to coating of transwell insert membranes, Matrigel was transported on ice to the laminar flow hood to prevent gelling. All Matrigel aliquoting and setup work with the Matrigel was done with cold PBS and transwell inserts and pipet tips were kept cold before use. For the preparation of the inserts, 120 µl of Matrigel was poured on to the inserts utilizing 1000 µl tips and the inserts incubated at 37 °C for 30 min to promote gelling.

Villi fragments were cut in a tissue culture hood and placed in a drop of PBS on aluminum foil pieces to aid tissue weight determination. All chorionic villi samples utilized were between 20-40 mg in wet weight. The villi fragments were placed on millicell-CM culture dish inserts pre-coated with Matrigel. Using a long needle, the fragments were spread out on the Matrigel to maintain their tree-like

architecture, but without pushing the fragments into the artificial substrate. The tissues were incubated for 30-60 min, without culture media, on the Matrigel to facilitate settling on the substrate. Serum free media was then added to each insert. For these particular experiments, DMEM/F12 media without phenol red containing 1 % Penicillin/Streptomycin and 0.25 μg/ml ascorbic acid, pH 7.4 was used. The wells of the tissue culture plate were also filled with DMEM/F12 media containing 10 % FBS as a source of growth factors to act as attractant and the explants were divided into experimental groups: four samples (n=4) were incubated at 3 % oxygen, 8 % oxygen and 20 % oxygen. The media was changed every 48 h and the experiments were conducted for 4-6 days in triplicate. Explants were fixed in 4 % PFA/PBS for 4 h at 4 °C, dehydrated with increasing concentrations of alcohol and then paraffin embedded.

2.14 RNA Isolation

RNeasy Mini Kit from Qiagen (Qiagen Cat # 74104) was used for preparation of RNA samples obtained from HTR8-SVneo cells cultured under normal and low oxygen environments. This method was used when the RNA was then analyzed by quantitative real time polymerase chain reaction (qRT-PCR) since the A260/A280 ratios obtained for RNA isolated with the TRizol method were not adequate indicating the quality of the RNA was not good enough for qRT-PCR.

Cells cultured in 6 well plates were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which

immediately inactivated RNases to ensure purification of intact RNA. Ethanol was then added to each cell sample to provide appropriate binding conditions, and each sample was applied to an RNeasy Mini spin column to bind total RNA while allowing efficient removal of contaminants. Several additional washing steps with different washing buffers ensured that high-quality RNA was then eluted in 20 µl of RNase-free water.

RNA was then quantified by absorbance measurement at 260 nm and the quality and integrity of the RNA was assessed by the A260/A280 ratio and electrophoresis on 1 % formaldehyde-agarose gels. RNA samples were then stored in the -80 °C freezer for future use.

2.15 Real Time PCR

2.15.1 DNase Treatment of the RNA

The purified RNA was treated with deoxyribonuclease I (DNase I, amplification grade, Invitrogen Cat # 18068-015) to remove any genomic DNA. Briefly, in RNase-free tubes, 1 µg of each RNA sample, 1 µl of 10X DNase I reaction buffer, 1 µl of DNase I (1 U/ µl) and DEPC-treated water to a final volume of 10 µl were mixed and incubated for 15 min at RT. DNase I was inactivated by the addition of 1 µl of 25 mM EDTA pH 8 followed by an incubation for 10 min at 65 °C. Samples were then used for qRT-PCR or stored at -80 °C.

2.15.2 First Strand cDNA Synthesis

Following DNase treatment, 8 µl of each RNA sample was mixed with 10 µl of 2X reverse transcriptase reaction mix containing oligo(dT), random hexamers, $MgCl_2$ and deoxyribonucleotides (dNTPs) followed by 2 µl of 2X reverse transcriptase reaction enzyme mix (SuperScript III First-Strand synthesis SuperMix for qRT-PCR, Cat # 11752-050, Invitrogen). This mix included the Moloney Murine leukemia Virus (M-MLV) reverse transcriptase that has been engineered to reduce RNase H activity and provide increased thermal stability. The reaction mix also included RNaseOut recombinant ribonuclease inhibitor to protect against degradation of RNA due to ribonuclease contamination. The total volume of each reaction was adjusted to 20 µl with the addition of DEPC-treated water and the reactions incubated at 25 °C for 10 min. Reaction tubes were then incubated at 50°C for 30 min and the reactions terminated by incubation at 85 °C for 5 min. Then 1 µl of E. coli RNase H was added to each tube and the mixtures incubated at 37 °C for 20 min. cDNA samples were either used for qRT-PCR or frozen at –20 °C until use.

2.15.3 Real Time PCR Analysis

qRT-PCR analysis was performed with the two step qRT-PCR kit from Invitrogen (Cat # 11765-100) using the SYBR® qPCR Supermix (Cat # 11760-100 Invitrogen). The following primers were utilized: ILK forward (5′-AAG CTG CTA

GTT CCA AGG AAC CCT -3′), ILK reverse (5′-TCC ATA CGG CAT CAT CCA GTG TGT GAT -3′); 18S forward (5'-GCG AAA GCA TTT GCC AAG AA -3′), 18S reverse (5′-GGC ATC GTT TAT GGT CGG AAC -3′). After an initial incubation of 50 °C for 2 min and 95 °C for 10 min the reactions were cycled 40 times. Each cycle consisted of denaturation at 95 °C for 15 s and primer annealing and extension at 60 °C for 30 s. Results were collected and analyzed with ABI 7000 software. The data were controlled for quantity of mRNA input by measuring the reference gene, 18S. The amplification efficiencies of the target (*ILK*) and reference gene (*18S*) were similar as calculated by a calibration dilution curve for each gene. The relative amount of *ILK* mRNA was calculated with the ΔΔCt method. Melting curves were checked to confirm specificity of the primers. Dissociation (melting) curves were performed at the end of each run to confirm specificity of the amplification. Experiments were performed in triplicate and repeated three times.

2.16 Statistical Analysis

Data from immunoblot band density, migration assays, invasion assays and MTT assays were analyzed using GraphPad Prism version 5.02 (GraphPad Software Inc; www.graphpad.com). A one-way analysis of variance (ANOVA) was performed on the data followed by a Tukey-Kramer or Newman-Keuls post-test. Data were considered significantly different when $P<0.05$.

CHAPTER 3

ILK IS EXPRESSED IN THE HUMAN PLACENTA AND REGULATES THE MIGRATION OF A TROPHOBLAST-DERIVED CELL LINE

3.1 ILK Expression in Human Placenta

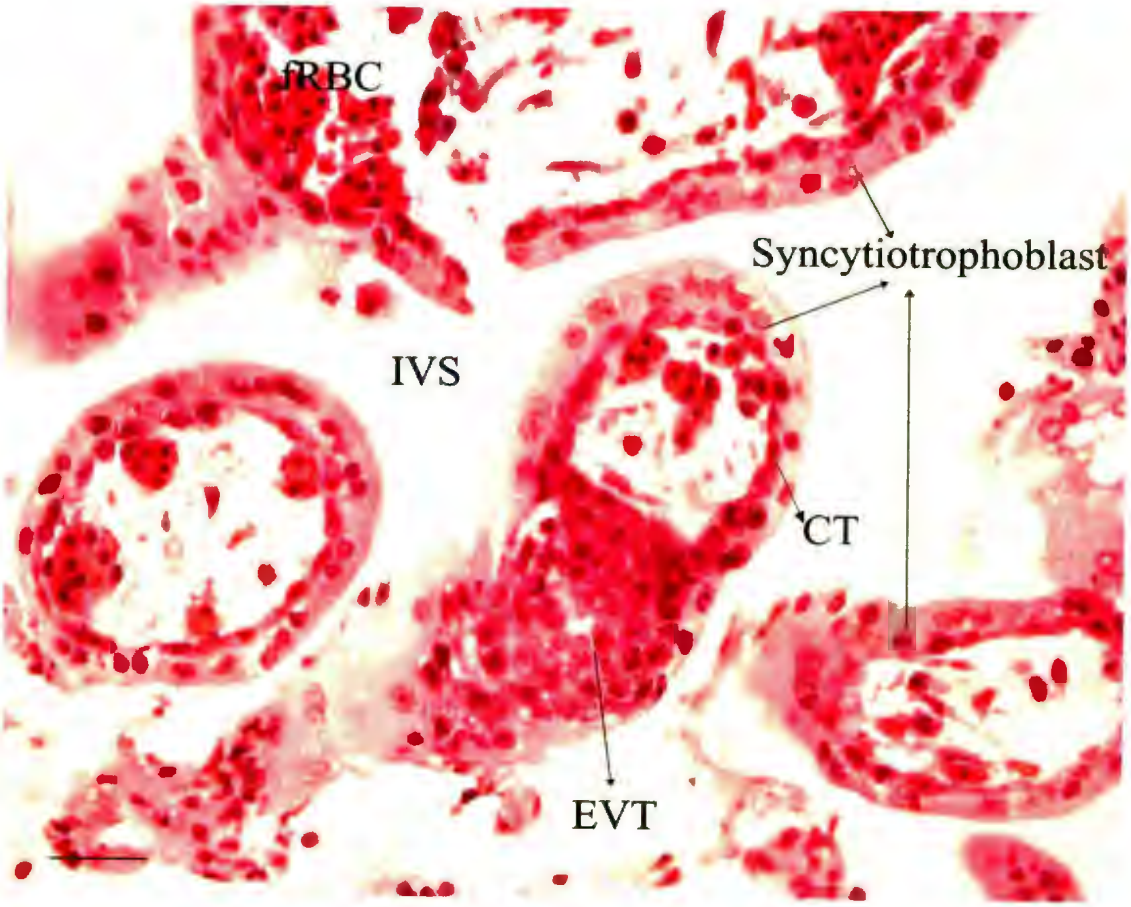
3.1.1 Experimental Rationale

ILK is overexpressed when tumors become invasive (Ahmed et al. 2003; Marotta et al. 2003; Ahmed et al. 2004). Since trophoblasts have an invasive nature, it was hypothesized that ILK plays an active role in the regulation of trophoblast differentiation from proliferative to invasive cells. To test this hypothesis, it was necessary to explore if ILK was expressed in human placental cells as well as where and when ILK was expressed during pregnancy. For this purpose, ILK expression was examined in human placentas collected from elective terminations and term deliveries.

3.1.2 The Structure of the Normal Chorionic Villi is Preserved Following Sample Preparation

To define the temporal and spatial pattern of ILK expression, human placentas from elective terminations were collected in a plastic trap after dilatation and curettage. As a consequence of this procedure the main organization of the organ was disrupted. A careful inspection of the tissue under a stereo-microscope ensured that only intact villi were collected in vials for fixation with 4 % PFA or for freezing in liquid nitrogen for storage. Following fixation and embedding, some paraffin sections were stained with hematoxylin and eosin to ensure that the different layers of the tissue were well preserved following sample collection (Fig 3.1). To properly identify the location of ILK in the human placenta, different markers were utilized such as cytokeratin 7 (CK7), human chorionic somatomammotropin (CSH1), αv-integrin (ITGAV), ITGA5, and von Willebrand factor (vWF). CK7 was used as a specific marker for both vCT and EVT (King et al. 2000) while CSH1, formerly known as human placental lactogen, was used as a marker for syncytiotrophoblast since it is secreted exclusively by the syncytiotrophoblast and exerts a metabolic effect on the mother to ensure that the nutritional demands of the fetus are met (Creasy and Resnik 2000). ITGAV heterodimerizes with integrin β5 and β6 in the vCT and in the EVT cells that are at the base of the cell column. They form the receptor for vitronectin and fibronectin (Huang et al 1998; van der Flier and Sonnenberg 2001). ITGA5 is a component of the receptor for fibronectin and is only expressed in those EVT that are becoming less proliferative and more invasive

Figure 3.1 A first trimester human placenta tissue section stained with Hematoxylin and Eosin. A representative photograph of week 9 placenta shows anchoring villi stained with Hematoxylin and Eosin. The syncytiotrophoblast, the cytotrophoblasts (CT) and the extravillous trophoblast (EVT) are indicated with arrows. The maternal blood occupies the intervillous (IVS) space during gestation but is lost after washes and fixation of the tissue. The stroma is composed of connective tissue and fetal blood vessels containing the nucleated fetal red blood cells (fRBC) that exchange gases and nutrients with the maternal blood. Scale bar, 50 μm.


fRBC
Syncytiotrophoblast
IVS
CT
EVT

(Fisher and Damsky 1993; Damsky et al. 1994; Zhou et al. 1997; van der Flier and Sonnenberg 2001). vWF is a specific marker of endothelial cells (Wagner et al. 1982; Warhol and Sweet 1984).

3.1.3 ILK is Present in Floating and Anchoring Villi of Human Placenta

Immunofluorescence analysis on the same placental sections corresponding to different weeks of gestation was used to identify those cells that expressed ILK. When ILK-specific antiserum and marker-specific antiserum were produced in the same species, adjacent serial sections were utilized for the study. The human placental samples were collected after informed written consent and ranged from week 6 to 12 in the first trimester (n=17), from week 13 to 15 (n=6) representing the early second trimester and from week 38 to 42 of gestation (n=10) at term. The immunofluorescence analysis of the different samples showed that ILK was highly detectable in floating branches of chorionic villi. Specifically, ILK was localized to cells of the villous stroma and to cytoplasm and plasma membranes of vCT (Fig. 3.2). The localization of ILK to the vCT was confirmed by the localization of the cytotrophoblast marker CK7 to the same location in adjacent sections (Fig 3.3).

Figure 3.2 Immunolocalization of ILK in floating branches of chorionic villi. Representative images of ILK expression in floating villi of human placenta from weeks (w) 6, 8, 10, 14, and 41 (term) of gestation. During the first and early second trimester, ILK was highly expressed in the villous cytotrophoblast layer but was also detectable in cells of the villous stroma and barely detectable in the syncytiotrophoblast layer. In term placenta, ILK was primarily localized to endothelial cells of fetal blood vessels. S: stroma; CT: villous cytotrophoblast; ST: syncytiotrophoblast; Control: non-specific mouse IgG control (w38). Scale bar, 50 μm.

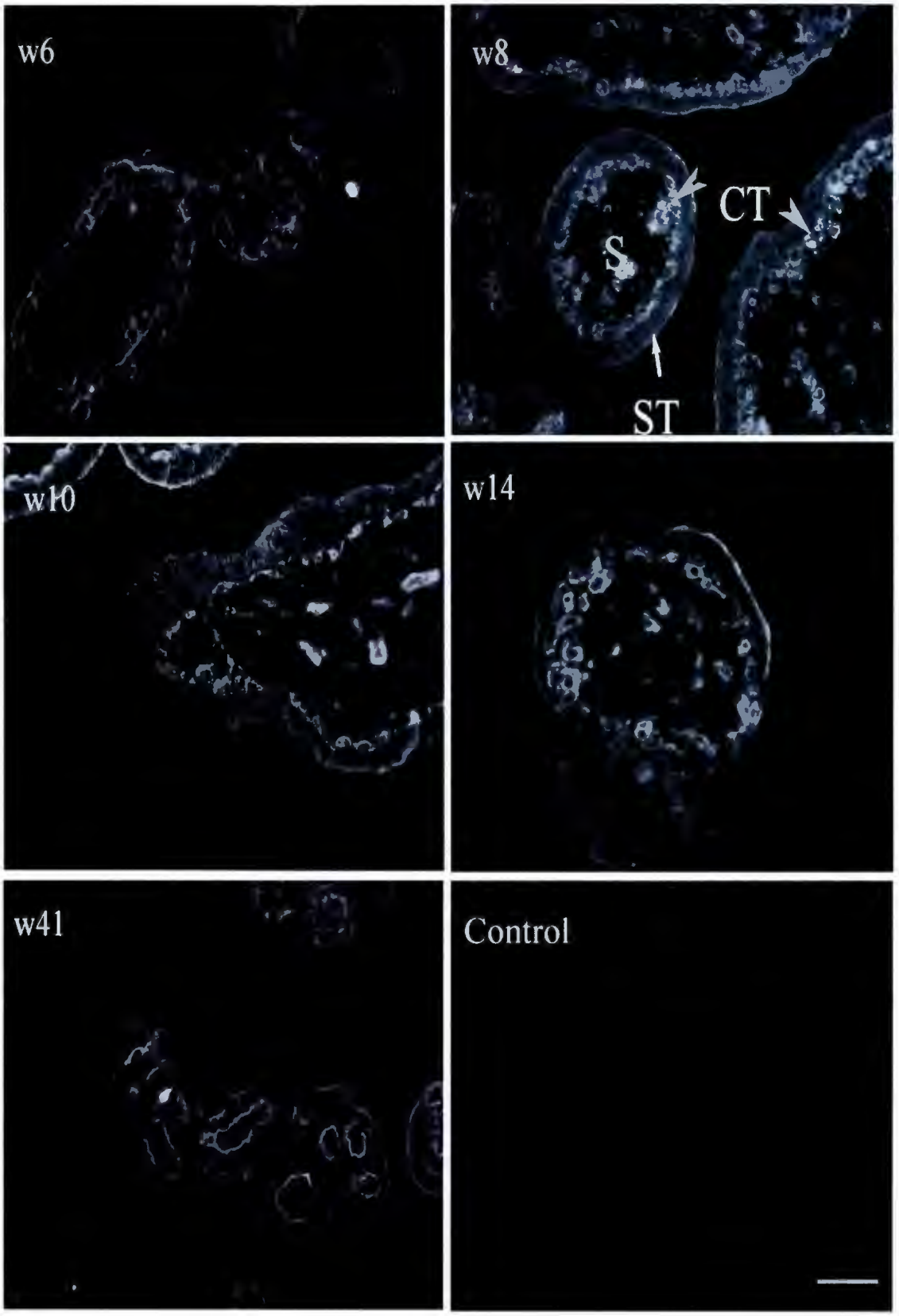
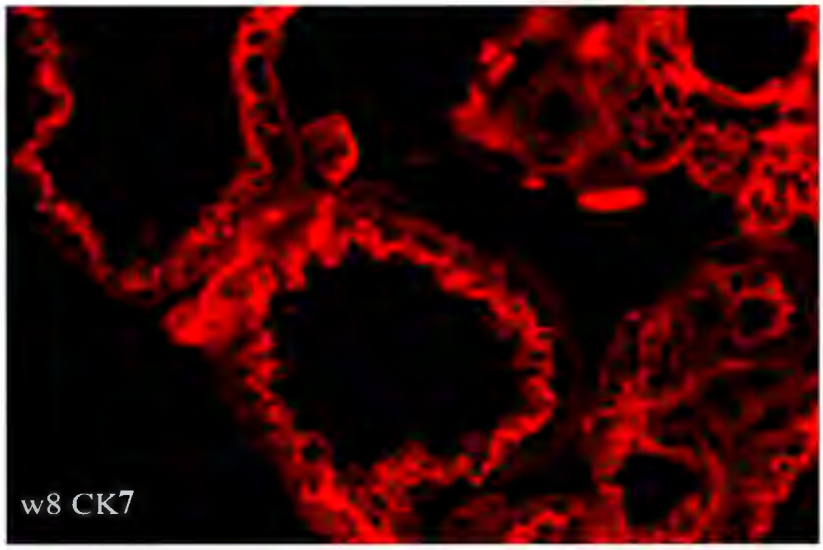
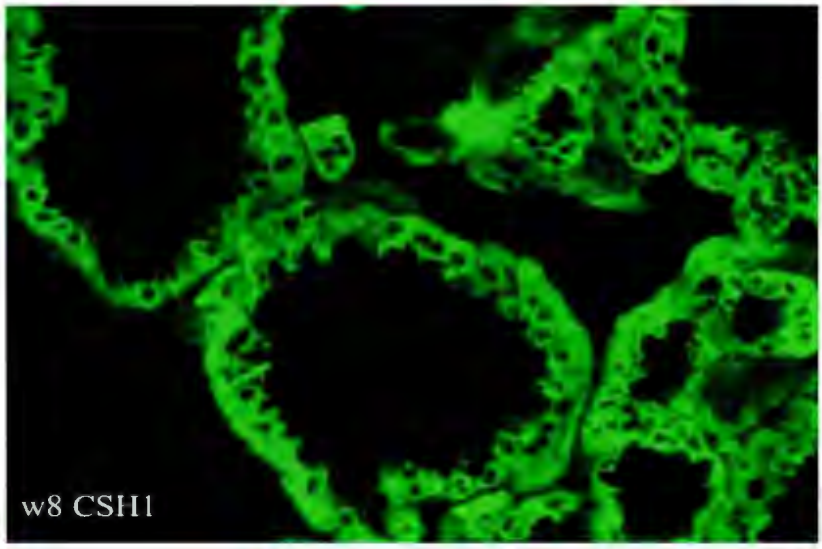
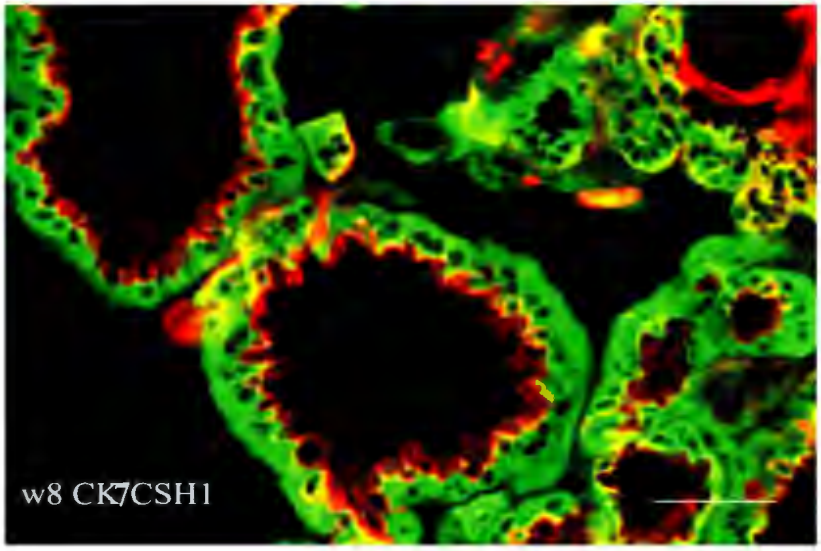

w6
w8
CT
S
ST
w10
w14
w41
Control

Figure 3.3 Human chorionic somatomammotropin and cytokeratin immunostaining of human placental villi. The syncytiotrophoblast was identified by immunostaining with an antibody specific for human chorionic somatomammotropin (CSH1; green). The underlying layer was identified by immunostaining with an antibody against cytokeratin 7 (CK7), a marker of cytotrophoblast (red). Shown is a representative human placental tissue section from week (w) 8 of gestation. CT: villous cytotrophoblast; ST: syncytiotrophoblast. Scale bar, 50 μm.


w8 CK7


w8 CSH1


w8 CK7CSH1

Compared to vCT, ILK was barely detectable in the overlying syncytiotrophoblast that was identified by immunostaining with a CSH1-specific antibody (Fig 3.4). The immunostaining of the syncytiotrophoblast layer with ILK antibody showed consistently similar intensity to the non-specific mouse IgG controls. In some sections, ILK was also detected, at a low level, on the apical surface of the syncytiotrophoblast layer. This result may be artifactual because of the presence of microvilli in this layer. It is possible that some residual non-specifically bound primary antisera remained trapped in proximity of these structures despite washes during the experiments, although the presence of ILK in this region cannot be ruled out completely.

In anchoring villi, ILK was primarily localized to the plasma membranes of EVT cell columns (Fig 3.5). Specifically, in proximal EVT of the cell column, ILK expression co-localized with ITGAV expression (Fig 3.6), whereas ILK co-localized with ITGA5 in more distal regions of the cell columns (Fig 3.7). When compared among different gestational time points, there was no detectable variation observed in the level of ILK expression *in situ*. The spatial pattern of expression was also similar in the different weeks of gestation analyzed, thus ILK was detected in the cytoplasm and plasma membrane of the vCT, in the mesenchymal cells of the chorionic villi and in EVT. The detection of ILK seemed lower in the vCT at term but this might only be a consequence of a lower number of vCT cells present in the floating villi.

From 6 to 15 weeks of gestation, ILK was also readily detectable in developing blood vessels in chorionic villi, specifically located in endothelial cells, as confirmed by co-immunolocalization of ILK with vWF (Fig 3.8). Similarly, in term

Figure 3.4 Immunolocalization of ILK and human chorionic somatomammotropin (CSH1) in human placenta. A representative tissue section of human placenta collected from week (w) 8 of gestation and immunostained with ILK and CSH1-specific antibodies. ILK was inconsistently detected, at a low level, on the apical surface of overlying syncytiotrophoblast cells that expressed the syncytiotrophoblast marker CSH1. CT, villous cytotrophoblast layer; ST, syncytiotrophoblast layer; S, stroma. Scale bar, 50 μm.

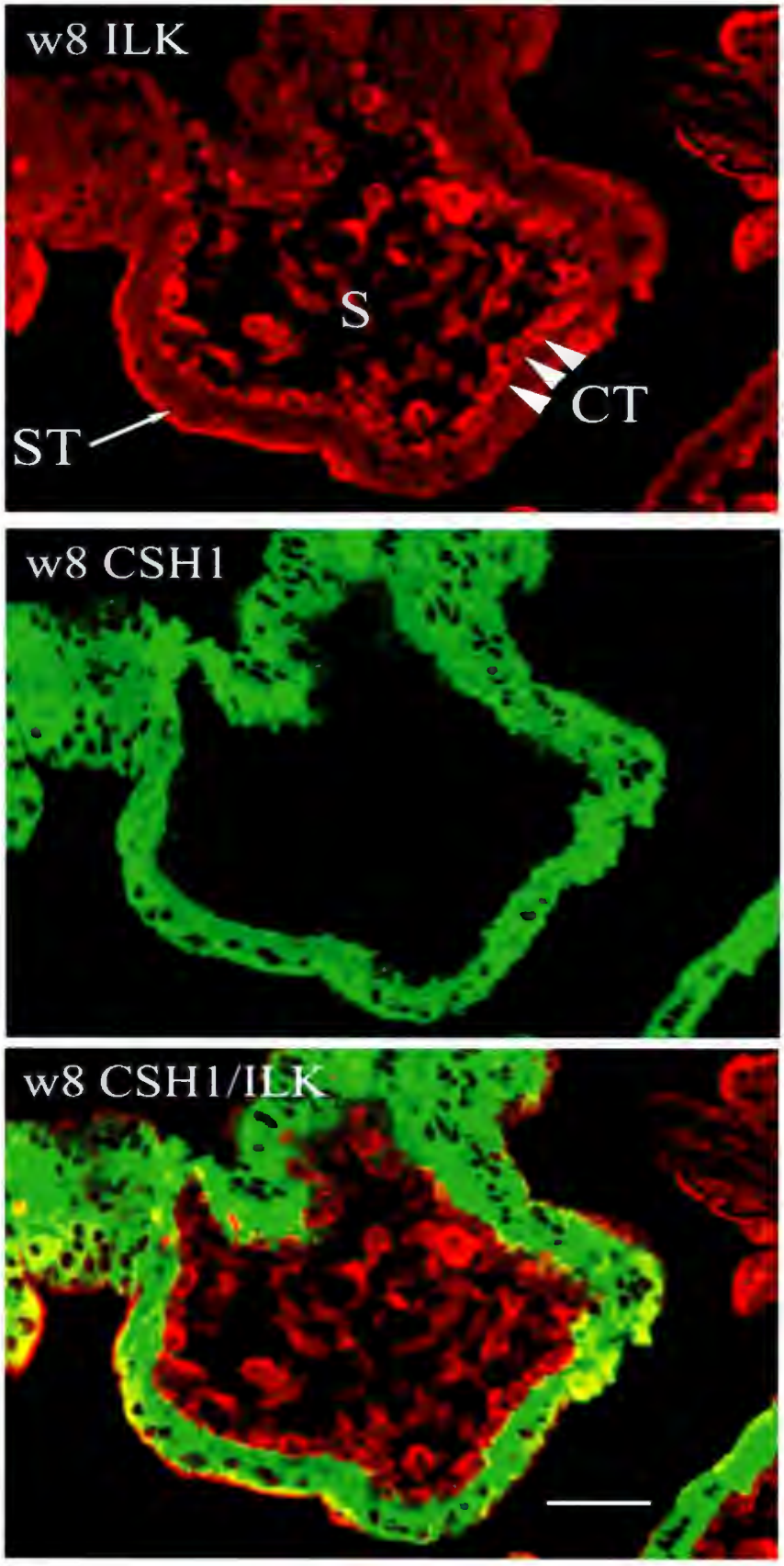

w8 ILK
S
CT
ST
w8 CSH1
w8 CSH1/ILK

Figure 3.5 Immunolocalization of ILK in anchoring branches of chorionic villi. Representative images of ILK expression in anchoring villi of human placenta from weeks (w) 6, 8, 11, 15, and 37 (term) of gestation. During the first and early second trimester, ILK was highly expressed in the villous cytotrophoblast and extravillous trophoblast but was also detectable in cells of the villous stroma and barely detectable in the syncytiotrophoblast layer. At term ILK was only detected in a few cells of the basal plate. EVT, extravillous cytotrophoblast; S, stroma; CT, villous cytotrophoblast; ST, syncytiotrophoblast; Control: non-specific mouse IgG control. Scale bar, 50 μm.

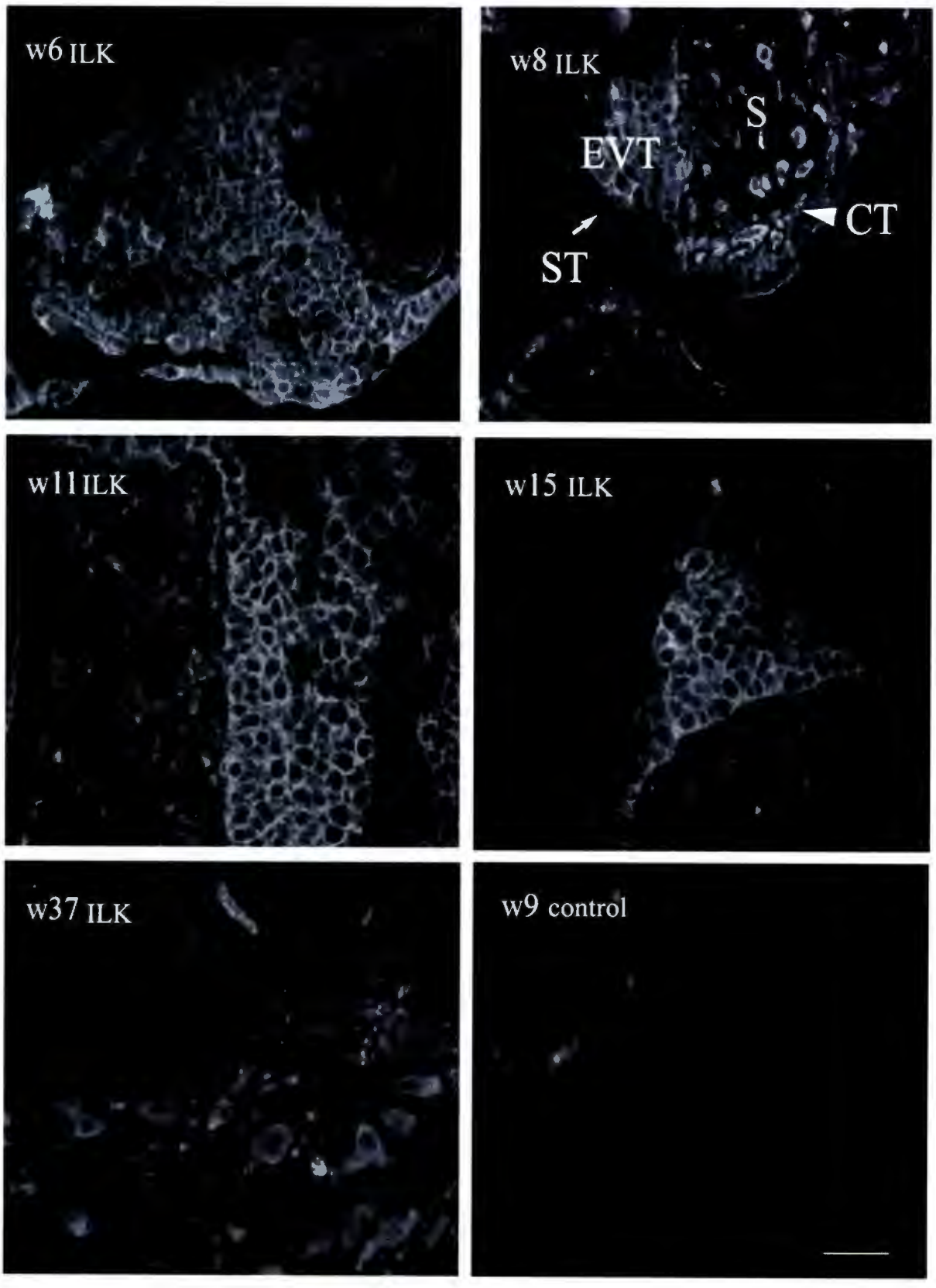
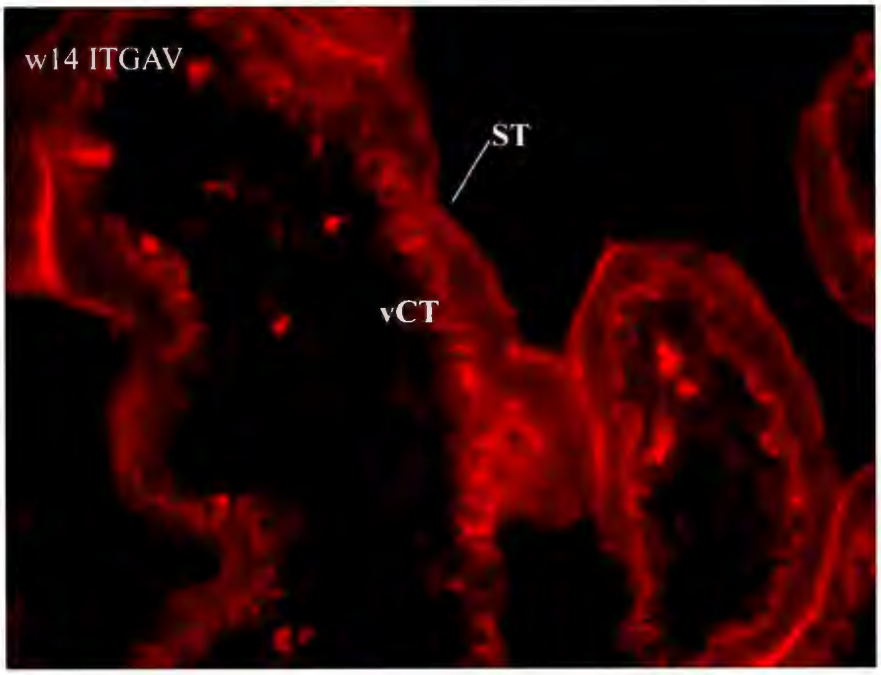

w6 ILK
w8 ILK
S
EVT
CT
ST
w11 ILK
w15 ILK
w37 ILK
w9 control

Figure 3.6 Expression of αv-integrin (ITGAV) in human placenta. Representative human placental tissue sections from week (w) 14 of gestation are shown. Indirect immunofluorescence analysis with floating (upper panel) and anchoring (lower panel) villi showed that ITGAV is expressed in the cells that form the base of the anchoring villi and in villous cytotrophoblasts where it demonstrates a plasma membrane localization. There was also slight detection of ITGAV in the apical aspect of the syncytiotrophoblast. EVT, extravillous trophoblasts; ST, syncytiotrophoblast; vCT, villous cytotrophoblasst. Scale bar, 50 μm.


w14 ITGAV
ST
vCT

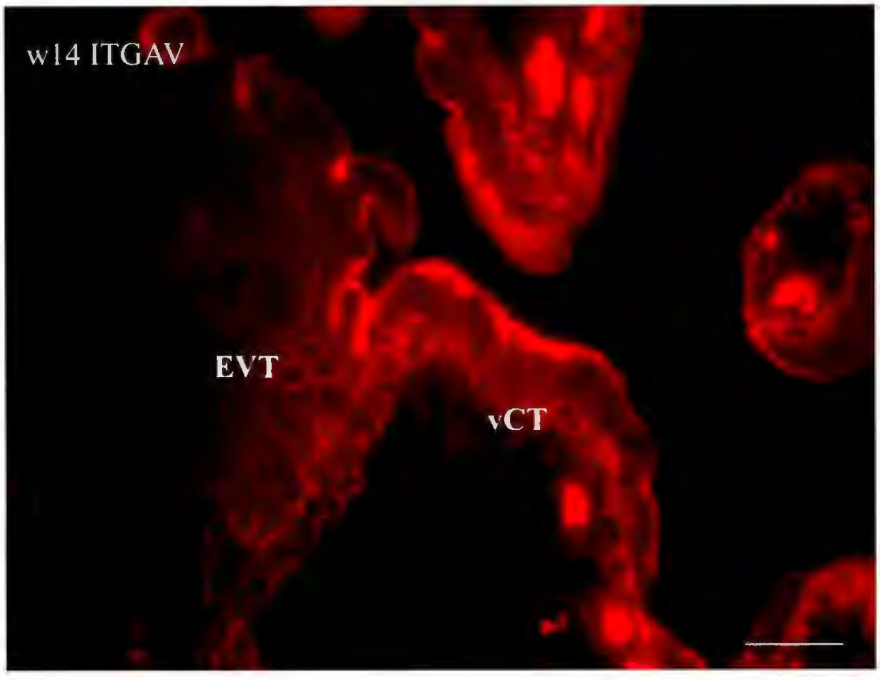

w14 ITGAV
EVT
vCT

Figure 3.7 Immunolocalization of ILK and ITGA5 in human placenta. Representative images from double immunofluorescence experiments with human placenta from week (w) 11 of gestation, illustrating that ILK co-localized (yellow) with ITGA5 in the EVT cell column. CT, villous cytotrophoblast layer; EVT, extravillous trophoblast; ST, syncytiotrophoblast layer; S, stroma. Scale bar, 50 μm.

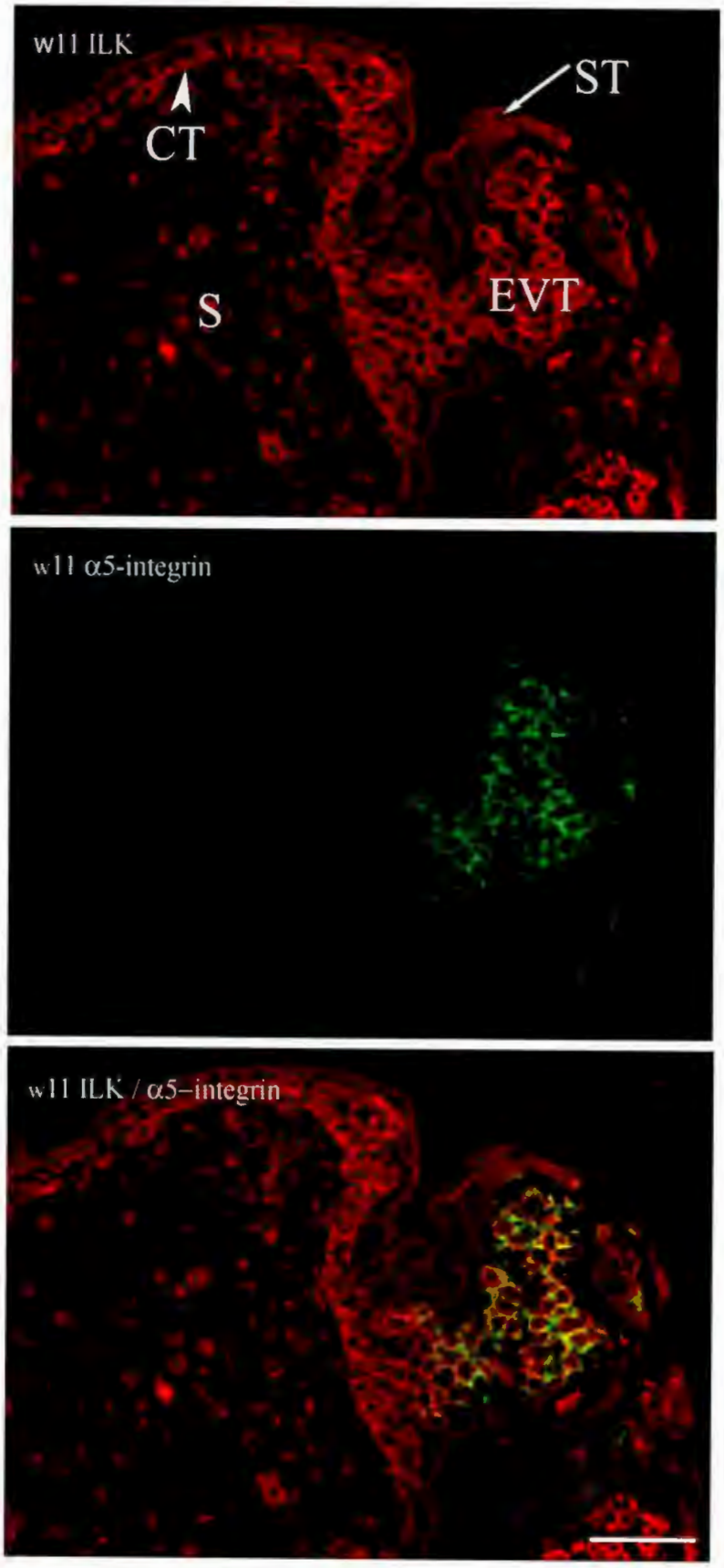
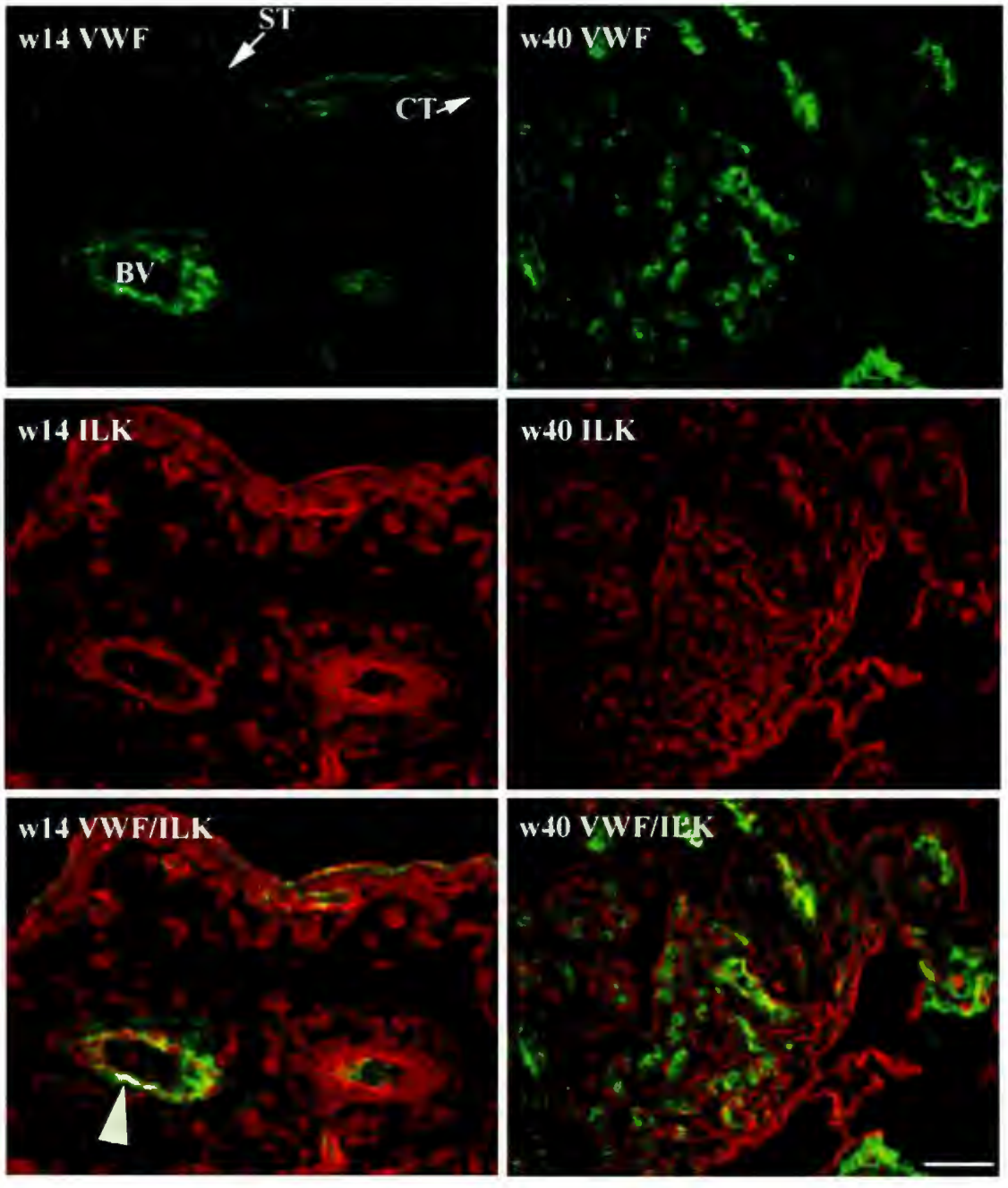

w11 ILK
CT
ST
S
EVT
w11 α5-integrin
w11 ILK / α5–integrin

Figure 3.8 Immunolocalization of ILK in blood vessels of chorionic villi. Representative images of chorionic villi from week (w) 14 and 40 of gestation illustrating the coimmunolocalization (arrowhead) of ILK with von Willebrand Factor (VWF) in blood vessel endothelial cells. BV, blood vessel; CT, villous cytotrophoblast layer; ST, syncytiotrophoblast layer. Scale bar, 50 μm.


w14 VWF
ST
CT
BV
w40 VWF
w14 ILK
w40 ILK
w14 VWF/ILK
w40 VWF/ILK

chorionic villi, ILK was still immunolocalized to endothelial cells of blood vessels, but markedly decreased in chorionic trophoblasts (Fig 3.8).

To establish whether there were any changes in the temporal pattern of ILK expression in placental tissue during the first trimester, early second trimester and at term, protein extracts from human placentas were studied by immunoblot analysis. This analysis demonstrated that ILK was readily and comparably detected, at the expected molecular weight of approximately 55 kDa at all gestational time points examined (Fig 3.9).

3.1.4 ILK Substrate, AKT, is Present in the Same Areas as ILK in the Human Placenta

AKT phosphorylation is regulated by ILK in many different cells such as PTEN null cancer cells (Wan and Helman 2003) and many reports showed that AKT phosphorylation on Ser-473 is dependent on ILK kinase activity (Persad et al. 2001; Nho et al. 2005). Thus, the temporal and spatial localization of Ser-473 phosphorylated AKT (pAKT) in first and early second trimester chorionic villi was studied to serve as an initial gauge of the activation state of ILK in this tissue. Immunoblot analysis demonstrated that pAKT was detectable, at the expected molecular weight of 60 kDa, in human chorionic villus tissue lysates at all gestational time points examined (Fig 3.10).

A lower molecular weight band of unknown identity was consistently detected in all placenta tissue lysates, but this may also potentially represent an AKT degradation

Figure 3.9 ILK protein expression in human placental lysates. A) A representative immunoblot showing ILK protein expression in human placentas in first trimester, early second trimester and at term. Placentas from weeks (w) 8, 9, 10, 12, 13 and 39 of gestation were studied by immunoblot analysis and showed comparable expression of ILK. β-Actin (ACTB) was used as the loading control. B) Densitometric analysis of immunoblots. The ILK and ACTB bands were densitometrically analyzed with Image J (NIH). ACTB bands were used to normalize the level of expression of ILK. ANOVA analysis ($P < 0.05$) was used to compare statistically the relative amounts of ILK in different weeks of gestation. No significant differences were found among the groups. Three sets of gestational profiles were studied (n=3).

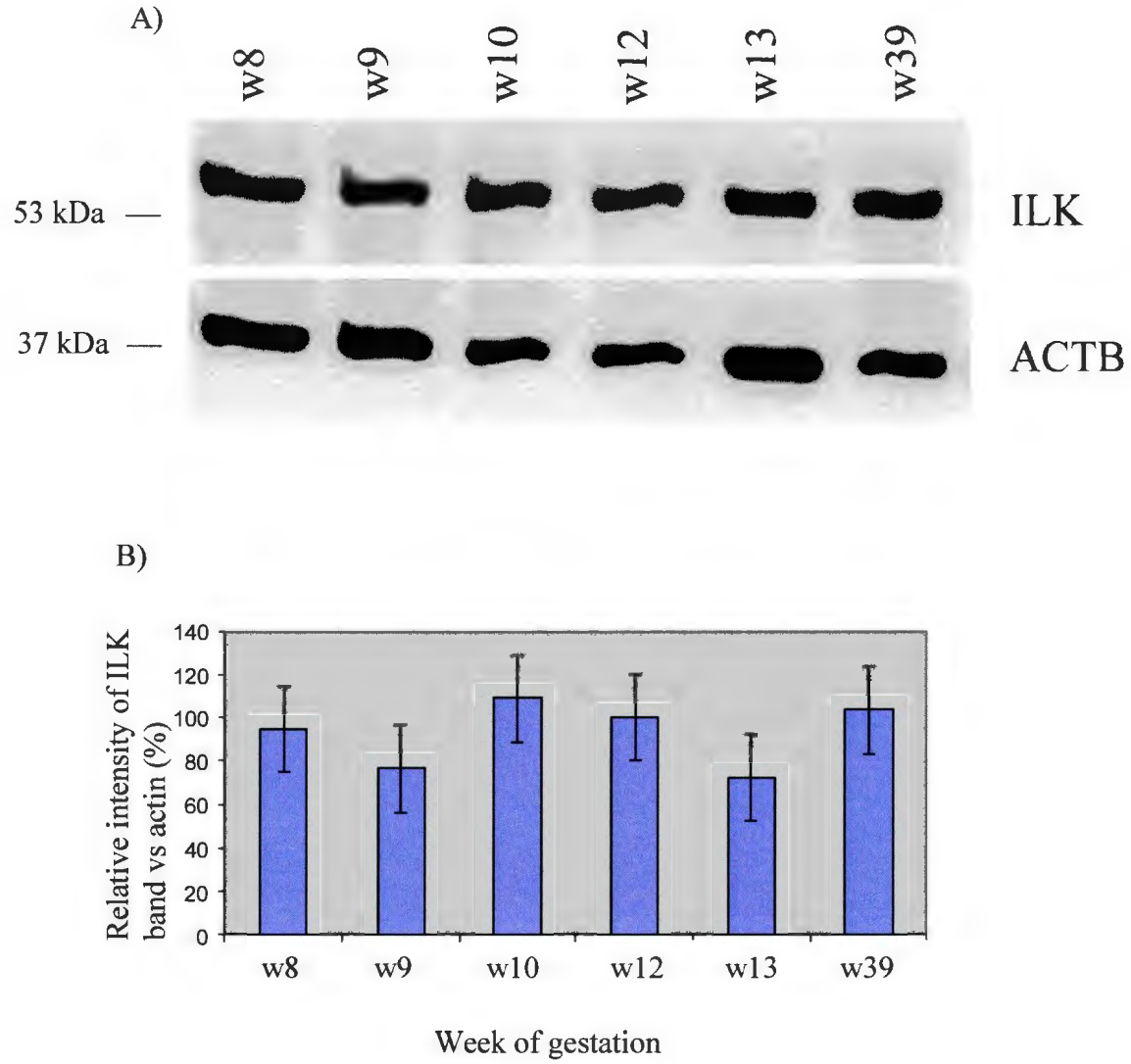
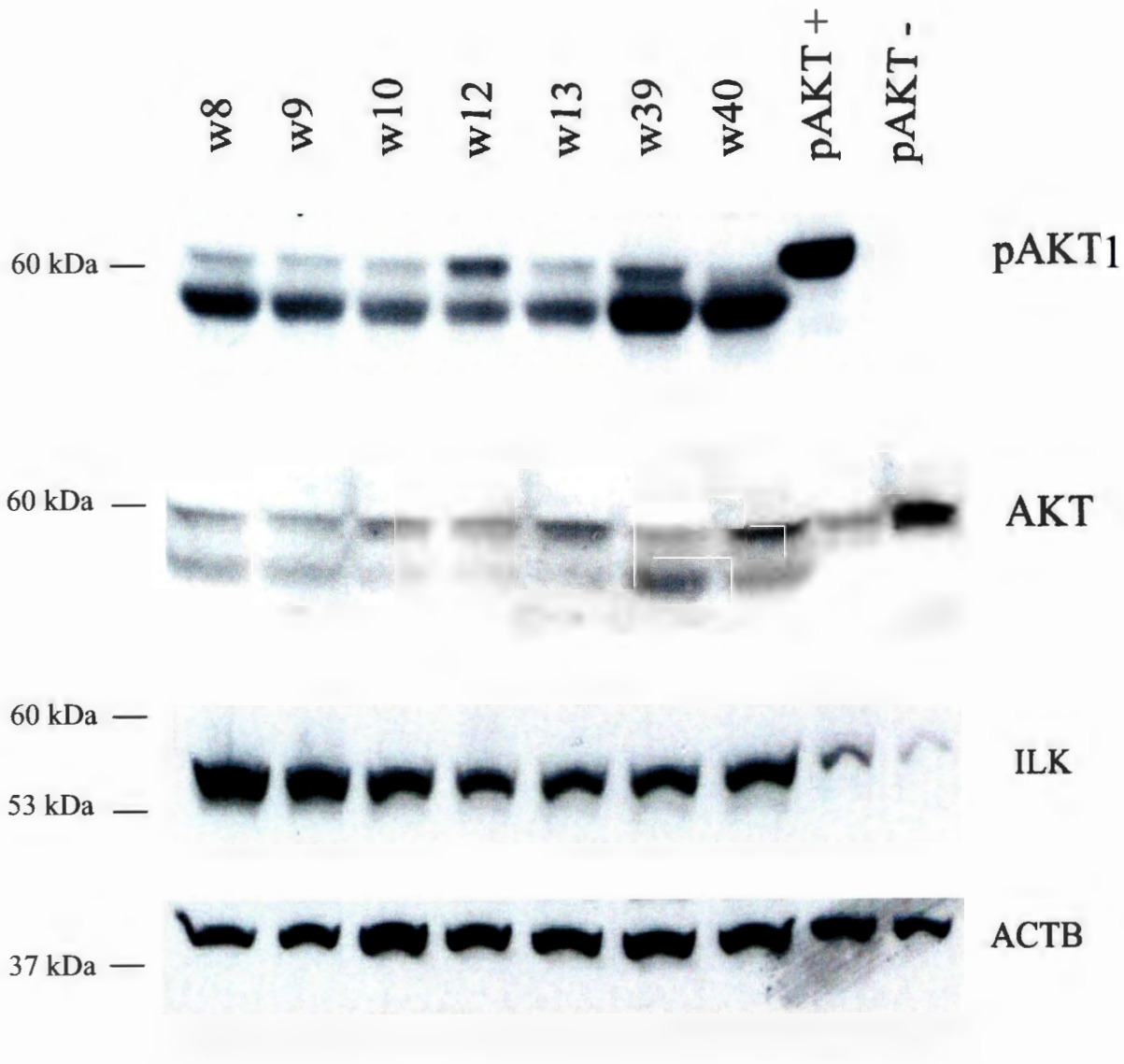

A)
w8
w9
w10
w12
w13
w39
53 kDa
ILK
37 kDa
ACTB
B)
Relative intensity of ILK band vs actin (%)
140
120
100
80
60
40
20
0
w8
w9
w10
w12
w13
w39
Week of gestation

Figure 3.10 Expression of AKT and pAKT in human placental lysates. AKT and pAKT are expressed in the same weeks (w) of gestation as ILK. Human placentas (w8, 9, 10, 12, 13, 39, 40) were studied by immunoblot analysis. Jurkat cell lysates of cells pre-treated (pAKT -) and untreated (pAKT +) with a PI3K inhibitor (LY294002) were included as negative and positive controls, respectively. β-Actin (ACTB) was used as the loading control. Two complete sets of different gestational profiles were studied (n=2).


w8
w9
w10
w12
w13
w39
w40
pAKT +
pAKT -
60 kDa
pAKT1
60 kDa
AKT
60 kDa
53 kDa
ILK
37 kDa
ACTB

product. Similarly to ILK immunolocalization, from week 6 to week 15 of human gestation, pAKT was also detectable in the villous stromal cells and cytoplasm of the villous trophoblast cells as well as associated with plasma membranes of the vCT and EVT cells (Fig 3.11). pAKT was virtually undetectable in the syncytiotrophoblast layer of the chorionic villi.

3.2 ILK Regulates Migration of the HTR8-SVneo Human Trophoblast Cell Line

The observation that ILK was expressed in those cells of the chorionic EVT column that were becoming less proliferative and more migratory and invasive, suggested that ILK might be involved in trophoblast cell migration. The role of ILK in migration was studied using a trophoblast derived cell line named HTR8-SVneo. The expression of ILK was first studied by immunofluorescence to make sure that ILK was expressed in these cells under normal culture conditions (RPMI 1640 media, 10 % FBS, 1 % Penicillin/Streptomycin, 5 % CO_2 in air) and low serum conditions (RPMI 1640 media supplemented with 0.5 % FBS). Under low serum conditions, the cells showed more intense staining at the plasma membrane compared to the rest of the cytoplasm (Fig 3.12). The nucleus showed the same staining level as the controls suggesting that there was no detectable amount of ILK in the nucleus of HTR8-SVneo cells but remarkably high levels of detection around the nucleus.

To examine whether or not ILK expression was comparable in the trophoblast derived cell line to other invasive cells, the relative amount of ILK expression was

Figure 3.11 pAKT expression in human placenta. These representative pictures of week (w) 9 and w14 placentas show that pAKT is expressed in human placenta in the same areas where ILK is expressed, the villous cytotrophoblast layer (CT) in the floating villi and in the extravillous cytotrophoblasts (EVT). The detection of pAKT was much lower in the syncytiotrophoblast where ILK was almost undetectable. ST, syncytiotrophoblast; S, stroma; Control: non-specific mouse IgG control. Scale bar, 50 μm.

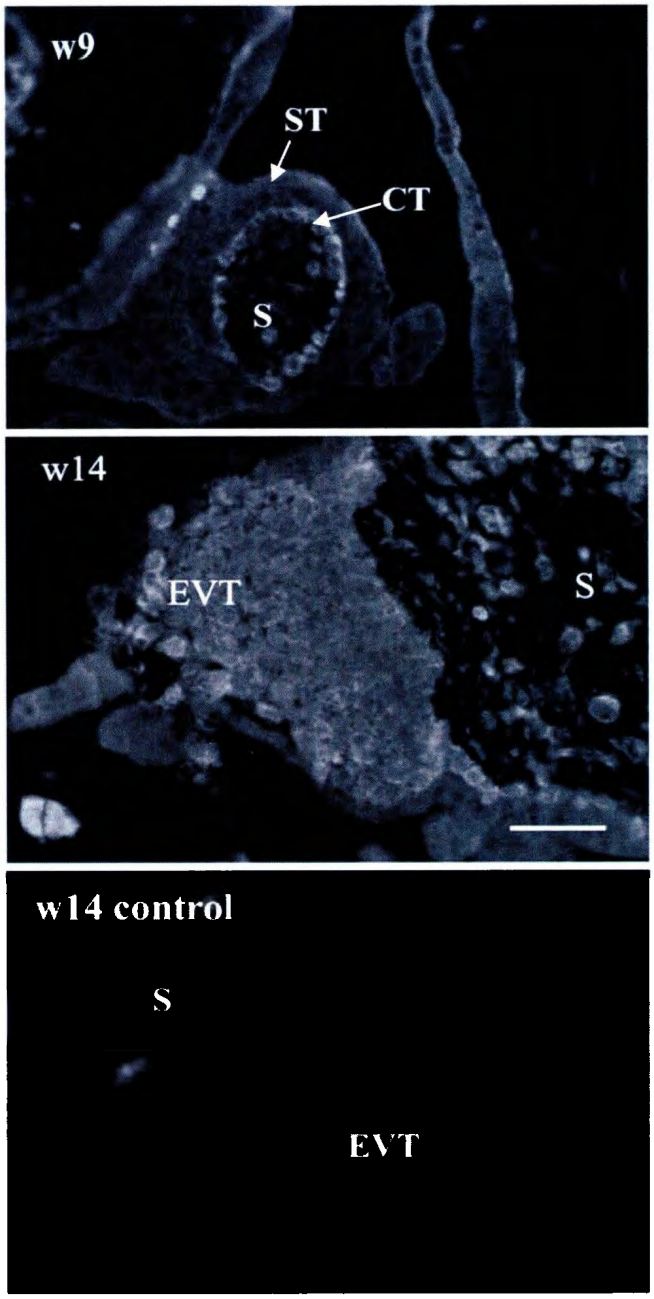
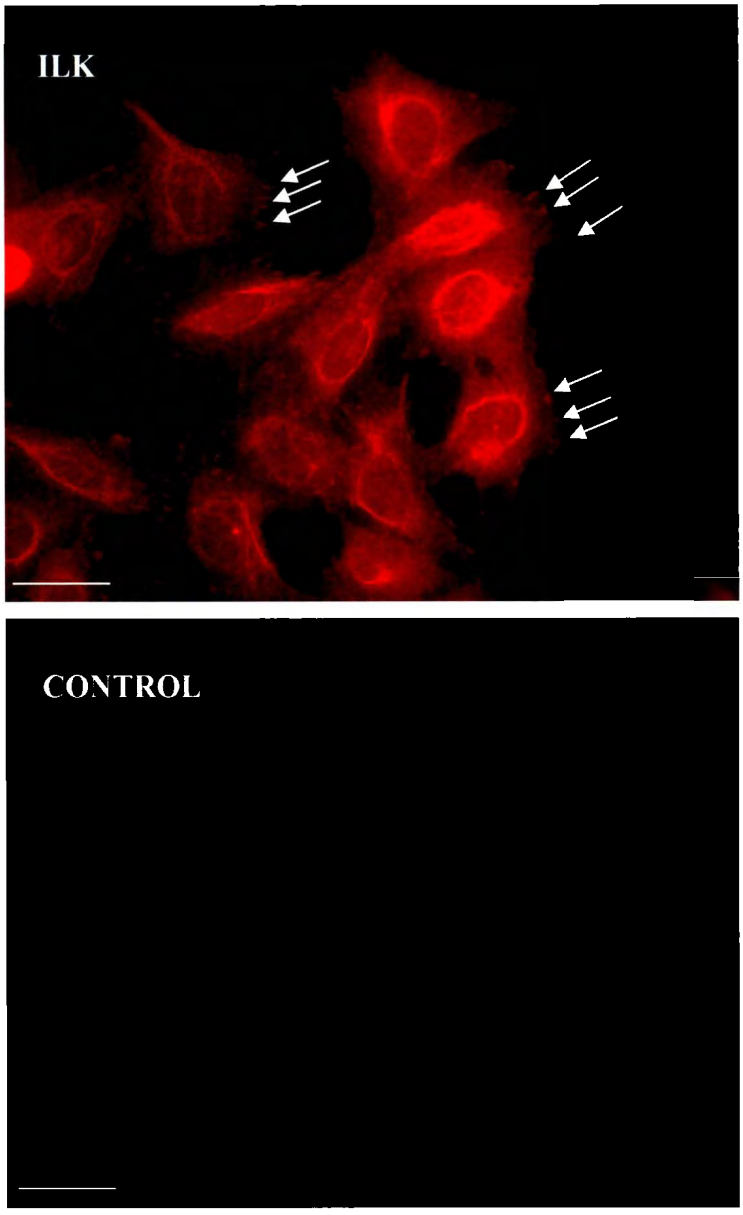

w9
ST
CT
S
w14
EVT
S
w14 control
S
EVT

Figure 3.12 ILK expression in HTR8-SVneo cells under low serum conditions. Cells were grown on coverslips in RPMI 1640 media supplemented with 0.5 % FBS for 24 h, then they were fixed and stained with an antibody raised against ILK or a non-specific mouse IgG control. The cells showed positive immunostaining for ILK in the cytoplasm with a stronger signal on the plasma membrane and around the nuclear membrane. At the plasma membrane ILK immunostaining suggested that it could be associated with focal adhesions (white arrows). Scale bar, 25 μm.


ILK
CONTROL

invasive such as cancer cells. Comparable levels of ILK were detected in HTR8-SVneo and 3T3 fibroblasts, mouse mammary epithelial (MME), cervical cancer (HeLa), and choriocarcinoma-derived cells lines, JEG-3 and JAR (Fig. 3.13).

3.2.1 Migration Assay

The literature indicates that ILK has a role in migration of different cells such as central nervous system cells (Belvindrah et al 2006), mammary epithelial cells (Filipenko et al 2005) and intestinal epithelial cells (Kim et al 2008) among others. Since in human placenta ILK was localized to those cells that express ITGA5 and are migratory, the role of ILK in migration was studied by transiently transfecting trophoblast derived cells, HTR8-SVneo, with different ILK containing plasmids (pEGFP-ILK-WT, pEGFP-ILK-E359K) or an empty vector (pEGFP) and performing migration assays. EGFP-ILK-E359K is an EGFP-fusion protein that has a mutation that results in a dominant negative form of ILK (Delcommenne et al 1998; Dedhar et al 1999).

First the expression of the plasmids in HTR8-SVneo cells was confirmed by immunoblot analysis. Transient transfection of pEGFP-ILK-WT and pEGFP-ILK-E359K for 48 h in HTR8-SVneo cells resulted in significant detection of the ILK-EGFP fusion proteins on immunoblots at the expected molecular weight of 80 kDa, unlike HTR8-SVneo cells containing empty pEGFP vector, thus confirming the presence of the expression vectors in the cells (Fig 3.14). Furthermore, the immunoblot analysis demonstrated that detection levels of EGFP-ILK-WT and

Fig 3.13 ILK protein expression in different invasive cell lines. A representative ILK immunoblot of the protein extracts of 3T3, JEG 3, JAR, HTR8-SVneo, HeLa and MME cells is shown. Jurkat cell lysates of cells pre-treated (pAKT -) and untreated (pAKT +) with a PI3K inhibitor (LY294002) were included as negative and positive controls, respectively. β-Actin (ACTB) was used as the loading control. The experiment was repeated twice (n=2).

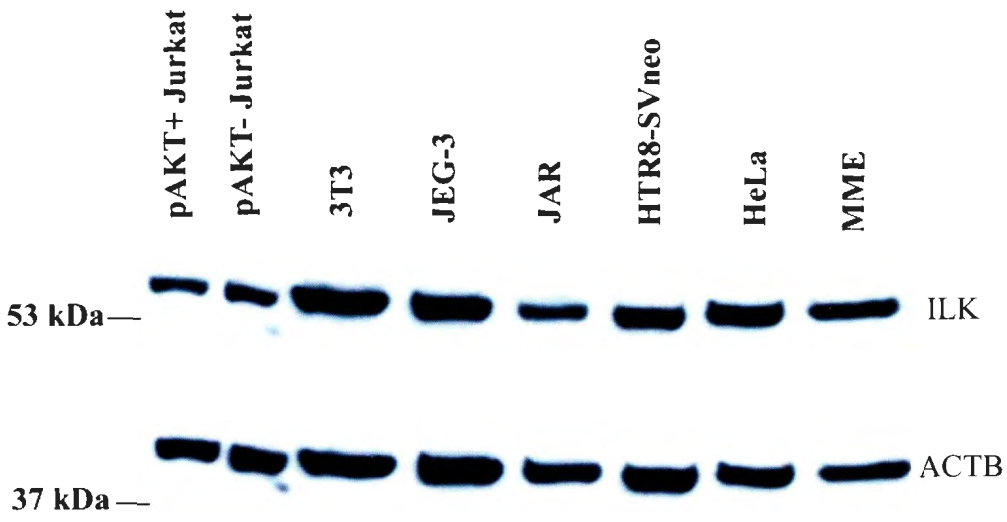
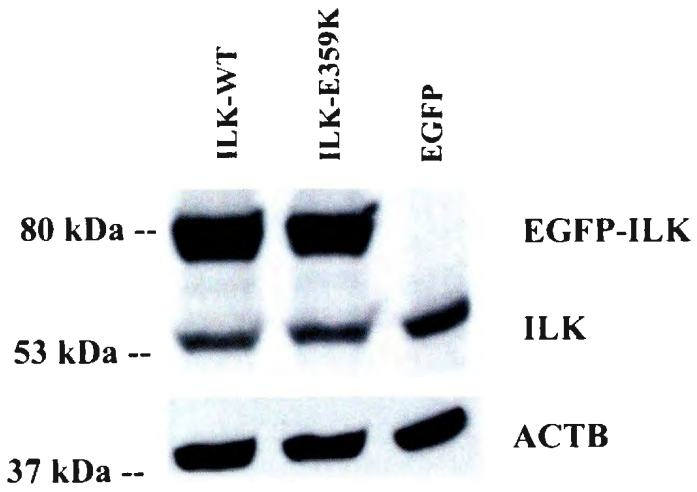

pAKT+ Jurkat
pAKT- Jurkat
3T3
JEG-3
JAR
HTR8-SVneo
HeLa
MME
53 kDa
ILK
37 kDa
ACTB

Figure 3.14 Immunoblot of protein extracts from HTR8-SVneo cells transiently transfected with the different ILK plasmids. Immunoblots were probed with ILK-specific antisera to detect endogenous ILK as well as EGFP-ILK fusion proteins. Transient expression of EGFP-ILK-WT and EGFP-ILK-E359K for 48 h in HTR8-SVneo cells subsequently resulted in significant detection of these fusion proteins on immunoblots at the expected molecular weight of 80 kDa. β-Actin (ACTB) was used as the loading control.


ILK-WT
ILK-E359K
EGFP
80 kDa --
53 kDa --
37 kDa --
EGFP-ILK
ILK
ACTB

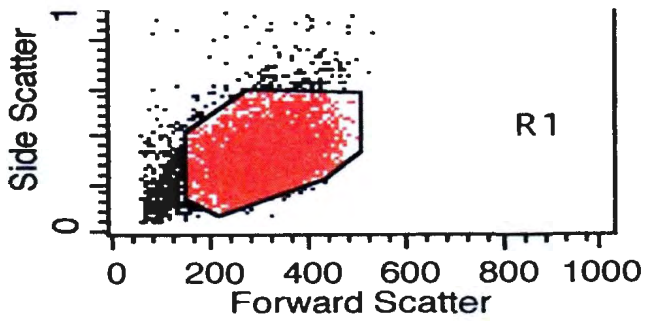
EGFP-ILK-E359K fusion proteins in the respective cell lysates were similar and did not affect the expression of the endogenous ILK. Routinely transfection efficiencies of ~40-50% were observed microscopically. If the efficiency appeared to be markedly lower, the cells were discarded and a new experiment was performed. There were no significant differences observed in the transfection efficiency of the cells with the three plasmids. A representative flow cytometry analysis also verified that the efficiency of the transient transfection was ~40% (Fig 3.15). The analysis was performed by Mrs. Kate Williams at the Electron Microscopy/Flow Cytometry Unit, Memorial University of Newfoundland. It is important to note that even though the transfection efficiency observed was similar in all experiments with the different ILK constructs, the intensity of the fluorescent signal was lower in the ILK-WT and the ILK-E359K-expressing cells when compared to the EGFP transfected cells. This may be a result of greater amounts of EGFP being produced in cells transfected with the empty vector compared to the production of larger EGFP-fusion proteins, which could be rate-limiting, upon transfection with the ILK-containing vectors.

When the migration assays were performed over a 24 h period, transient expression of dominant negative pEGFP-ILK-E359K in HTR8-SVneo cells dramatically reduced the migration of these EGFP-labeled cells into wounds compared to cells expressing pEGFP-ILK-WT or pEGFP vector itself (Fig 3.16 and 3.17; one-way ANOVA, $P < 0.05$).

MTT viability assays demonstrated that the observed reduction in cell migration of HTR8-SVneo cells expressing pEGFP-ILK-E359K was attributable to the effects of dominant negative ILK expression, because these cells did not display

Figure 3.15 Flow cytometry of cells transiently transfected with pEGFP. a) Acquisition dot plot showing population of cells gated (region R1). b) Acquisition histogram of the cells within R1 showing that 40 % of the cells were expressing EGFP. The number of cells is shown in the vertical axis and the intensity of the green fluorescence in the X-axis. This experiment confirmed that the efficiency for the transient transfection was 40 % (M1).

a)


Side Scatter
1
0
R1
0
200
400
600
800
1000
Forward Scatter

b)

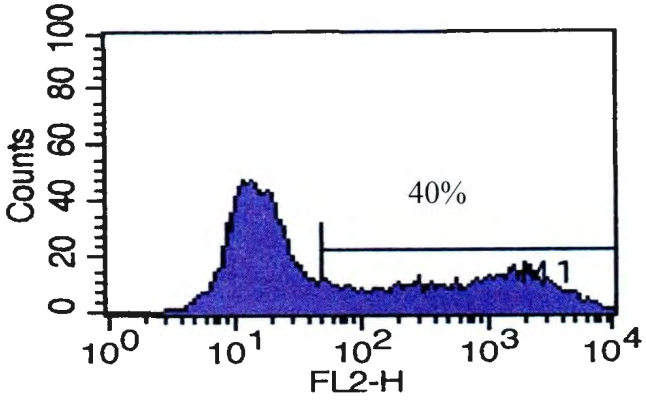

Counts
0
20
40
60
80
100
40%
M1
10^0
10^1
10^2
10^3
10^4
FL2-H

Figure 3.16 Migration assay on uncoated surface. Transient overexpression of pEGFP-ILK-E359K in HTR8-SVneo cells dramatically inhibits cell migration. Phase contrast and corresponding fluorescent images of EGFP from wound assays with HTR8-SVneo cells transiently overexpressing the EGFP-ILK fusion proteins are shown. HTR8-SVneo cells were transiently transfected with either pEGFP (empty vector; EGFP) or pEGFP vector containing wild-type (WT) ILK or dominant negative (E359K) ILK. Following creation of a wound with a pipette tip, the wound area was photographed (0 h). Cells were grown for 24 h in low serum conditions. Wounds were then photographed (24 h) and the number of EGFP-positive cells in the wound area counted relative to total cells in the wound. Yellow bars indicate the position of the wound in the monolayer. Scale bar, 100 μm. The experiment was repeated three times and conducted in triplicate (n=3).

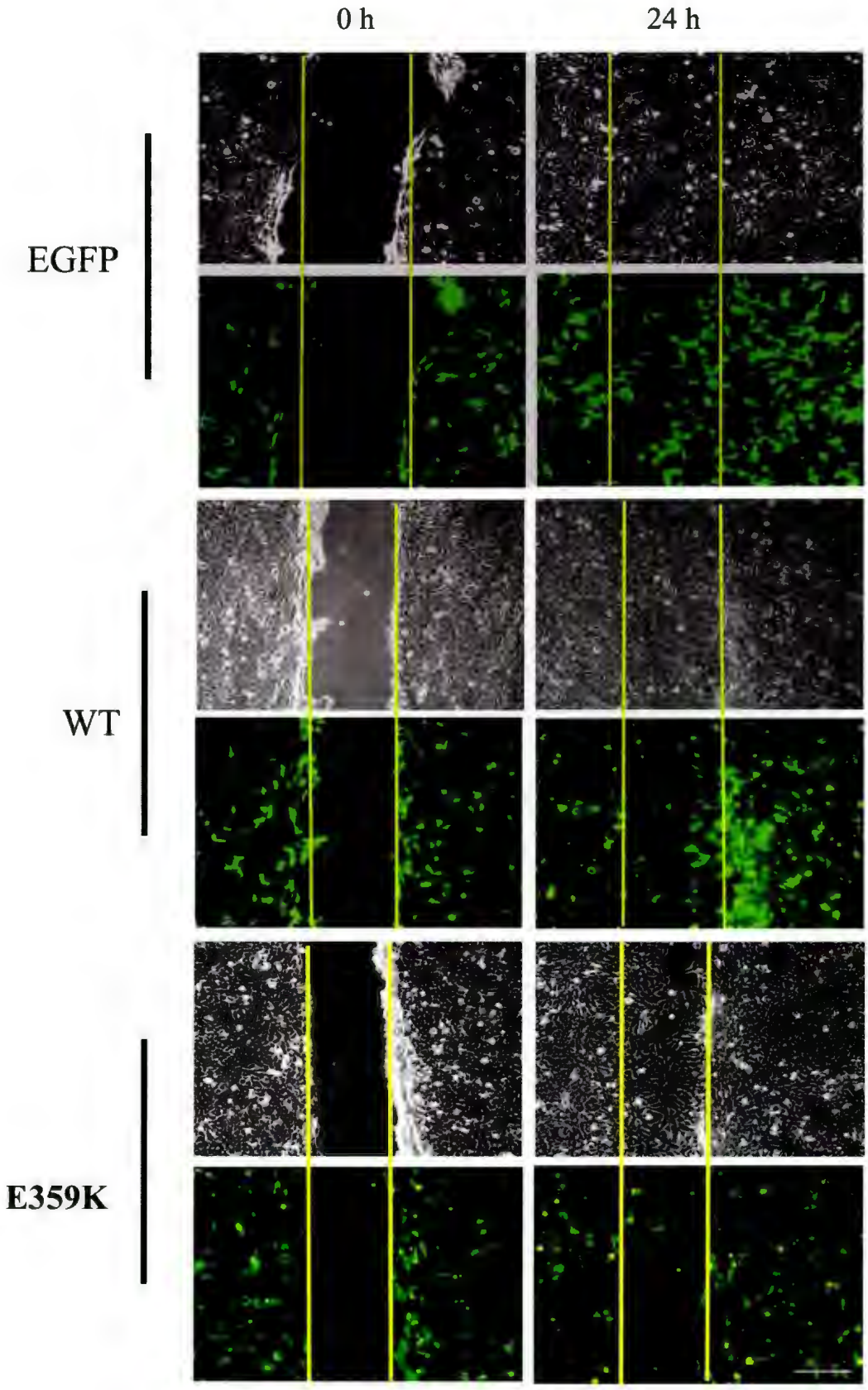
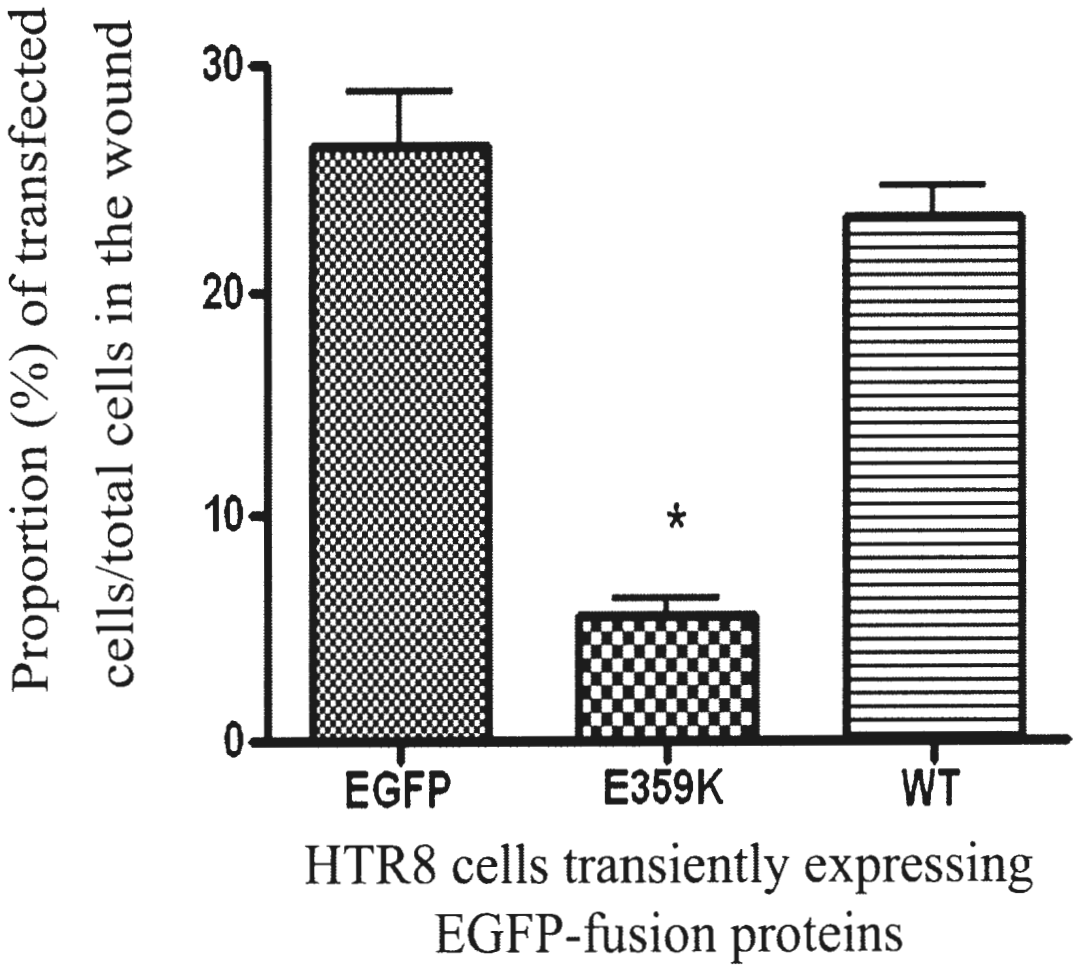

0 h
24 h
EGFP
WT
E359K

Figure 3.17 Quantitative analysis of wound assays with HTR8-SVneo cells transiently overexpressing EGFP-ILK fusion proteins. The number of EGFP expressing cells were counted in each photographed wound area and expressed relative to the total number of cells present in that wound area. Transient overexpression of dominant negative ILK-E359K (E359K) dramatically inhibited cell migration into the wound (n=3; ANOVA, $P < 0.05$; Tukey-Kramer post-test) compared to cells overexpressing wild-type ILK (WT) or empty vector (EGFP).


Proportion (%) of transfected cells/total cells in the wound
30
20
10
0
*
EGFP
E359K
WT
HTR8 cells transiently expressing EGFP-fusion proteins

any impaired viability as a result of pEGFP-ILK-E359K expression compared to pEGFP-ILK-WT or pEGFP- expressing cells (Fig. 3.18).

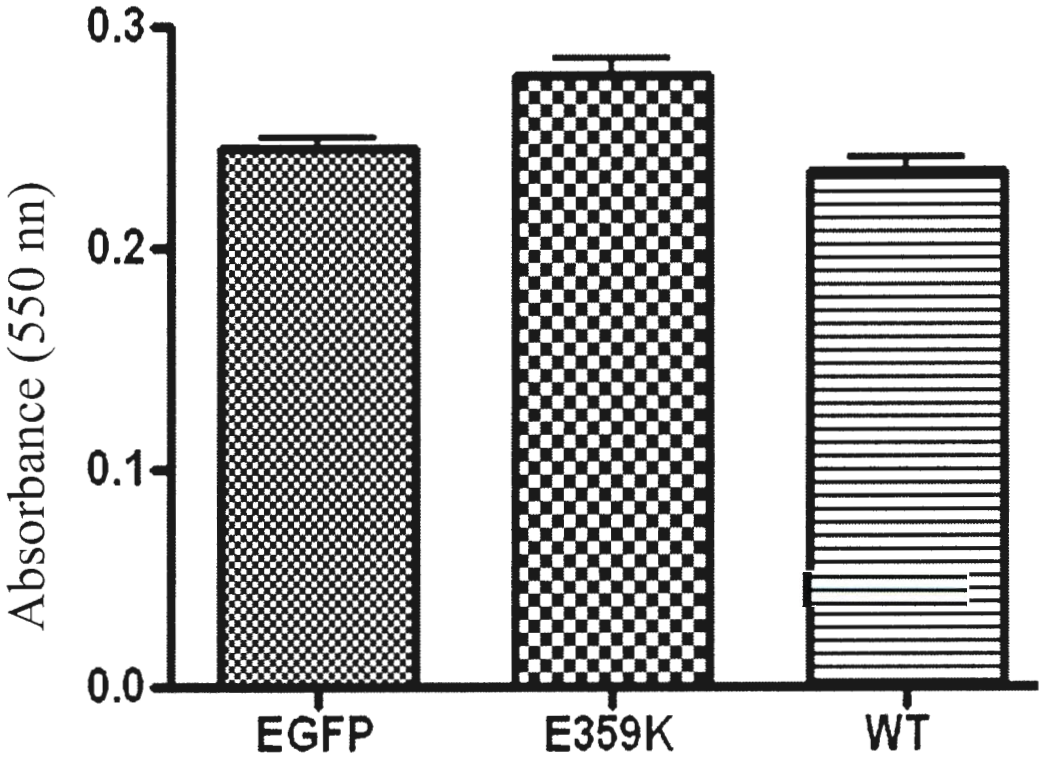
It is well known that the proteins of the extracellular matrix (ECM) regulate the integrin signaling pathway (Hynes 1992; Assoian and Marcantonio 1997; Hynes 2002). To test if the cells transfected with the dominant negative form of ILK were unable to migrate because there was no signal from the ECM, different matrices such as collagen, laminin and fibronectin were used to coat the slides on which the migration assays were performed. The experiments were performed in the same way as described above. Fewer ILK-E359K expressing cells were observed to migrate on the different matrices when compared with EGFP expressing cells (Fig 3.19-3.21). These results were consistent with the previous migration assay experiment on uncoated coverslips (Fig 3.16) indicating that the migration pattern of HTR8-SVneo cells transfected with dominant negative ILK was not due to the lack of ECM.

Attempts to determine if AKT phosphorylation was affected when the cells were transiently transfected with pEGFP-ILK-WT, pEGFP-ILK-E359K or pEGFP empty vector resulted in no evident effect of ILK fusion proteins on AKT phosphorylation (Fig 3.22).

3.2.2 ILK and Actin Organization

In order to determine what mechanisms might be involved in the regulation of

Figure 3.18 MTT assays. Transient overexpression of dominant negative ILK-E359K (E359K) or wildtype ILK (WT) in HTR8-SVneo cells did not significantly decrease cell viability/proliferation relative to HTR8-SVneo cells overexpressing EGFP. Columns represent averages ± SEM (one way ANOVA, $P < 0.05$; Tukey-Kramer post-test).



HTR8-SVneo cells transiently expressing EGFP-fusion proteins

Figure 3.19 Migration assay on collagen. HTR8-SVneo cells were cultured on collagen-coated coverslips to confluency. HTR8-SVneo cells were transiently transfected with either pEGFP (empty vector; EGFP) or pEGFP vector containing dominant negative (E359K) ILK. Twenty four hours after creation of a wound with a pipette tip, the cells were photographed. Phase contrast and corresponding fluorescent images of EGFP from wound assays with HTR8-SVneo cells transiently overexpressing the EGFP or EGFP-ILK-E359K proteins are shown. Fewer HTR8-SVneo cells migrated into the wound when they were transiently transfected with dominant negative pEGFP-ILK-E359K compared to cells transiently transfected with EGFP empty vector. Vertical bars indicate the position of the wound in the monolayer. The experiments were done in triplicate. Scale bars, 100 μm.

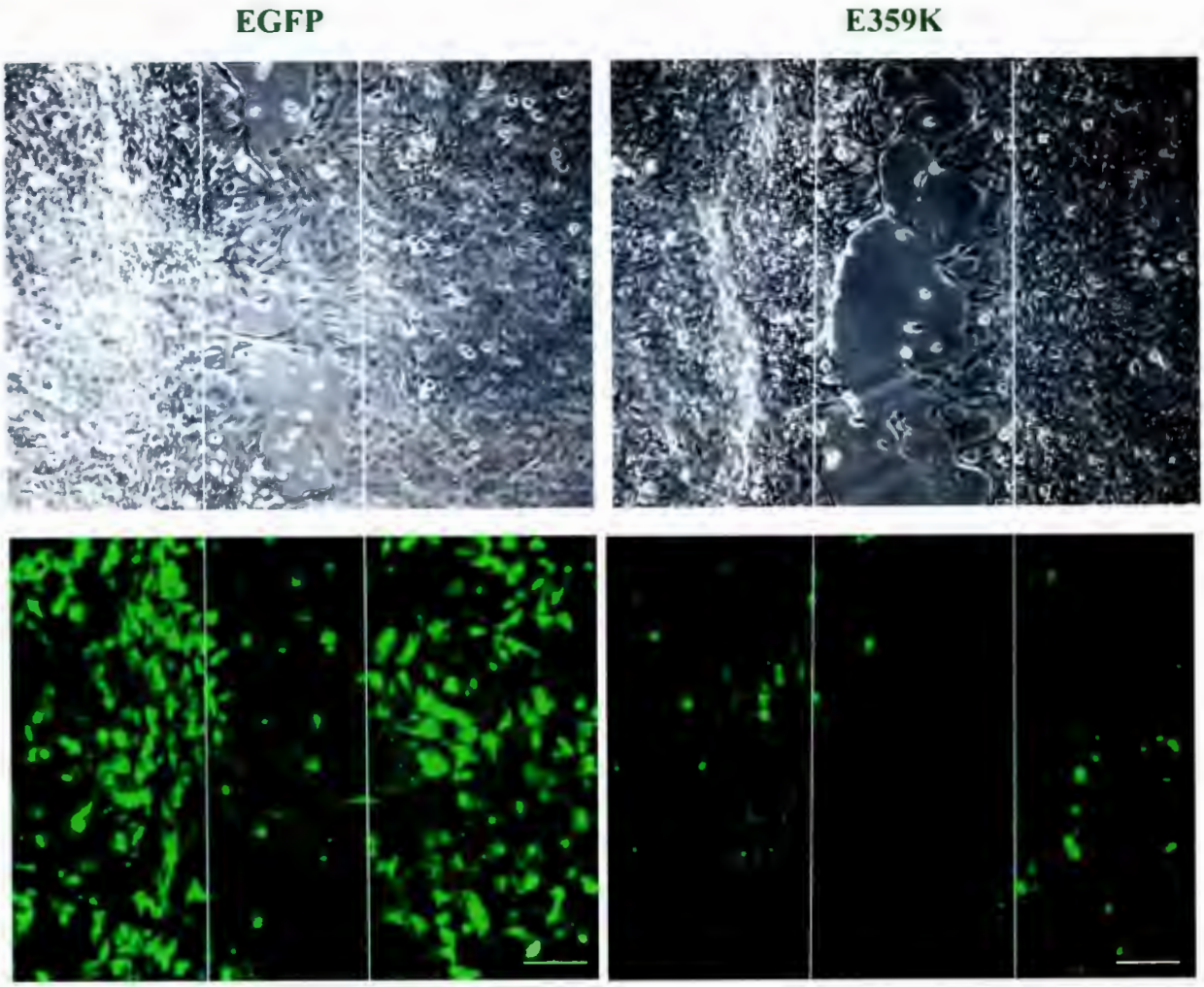

EGFP
E359K

Figure 3.20 Migration assay on fibronectin. HTR8-SVneo cells were cultured on fibronectin coated coverslips to confluency. HTR8-SVneo cells were transiently transfected with either pEGFP (empty vector; EGFP) or pEGFP vector containing dominant negative (E359K) ILK. Twenty four hours after creation of a wound with a pipette tip, the cells were photographed.. Phase contrast and corresponding fluorescent images of EGFP from wound assays with HTR8-SVneo cells transiently overexpressing EGFP or EGFP-ILK-E395K proteins are shown. Fewer HTR8-SVneo cells migrated into the wound when they were transiently transfected with dominant negative pEGFP-ILK-E359K compared to cells transiently transfected with EGFP empty vector. Vertical bars indicate the position of the wound in the monolayer. The experiment was done in triplicate. Scale bar, 100 μm.

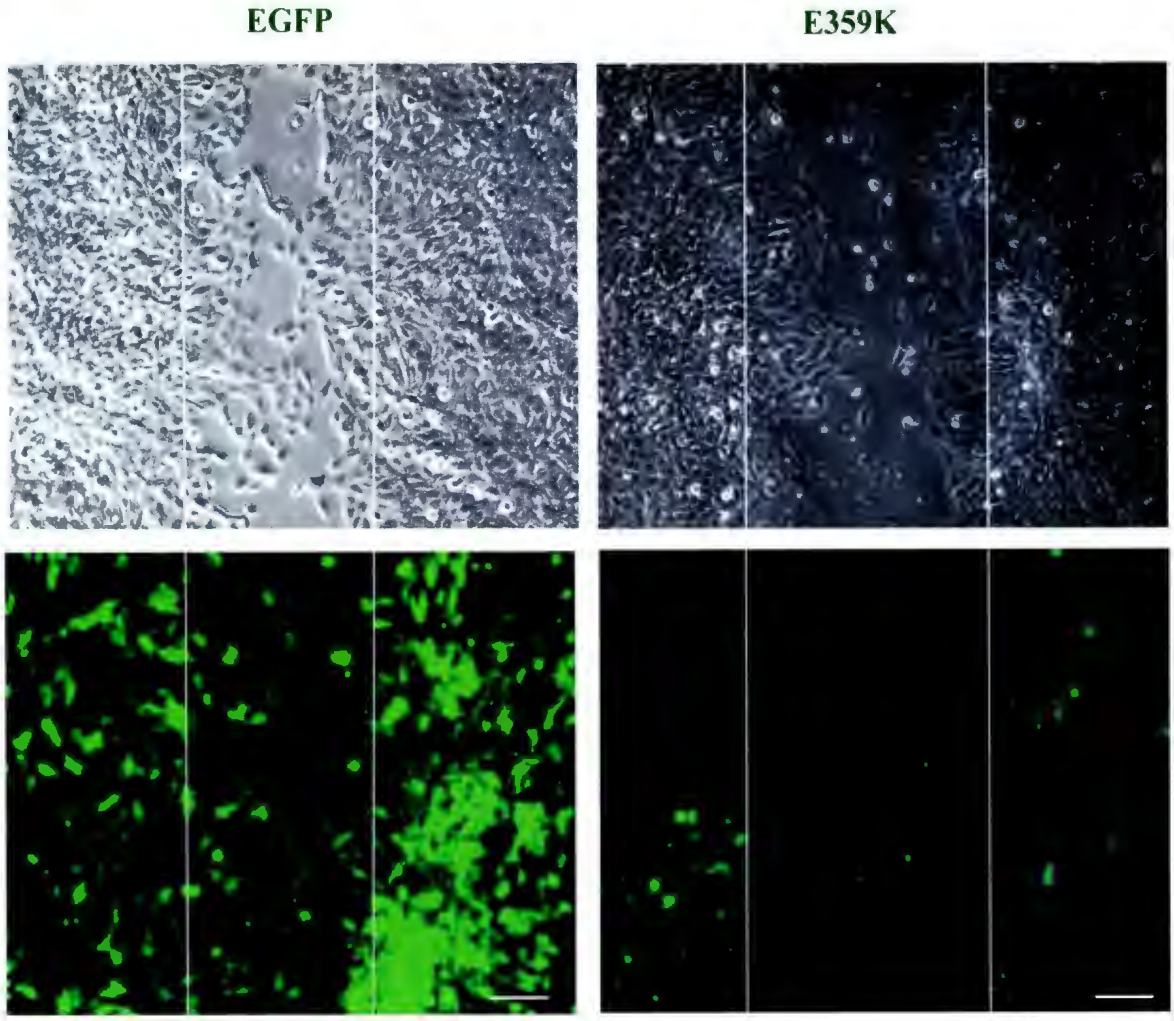

EGFP
E359K

Figure 3.21 Migration assay on laminin. HTR8-SVneo cells were cultured on laminin-coated coverslips to confluency. HTR8-SVneo cells were transiently transfected with either pEGFP (empty vector; EGFP) or pEGFP vector containing dominant negative (E359K) ILK. Twenty four hours after creation of a wound with a pipette tip, the cells were photographed. Phase contrast and corresponding fluorescent images of EGFP from wound assays with HTR8-SVneo cells transiently overexpressing EGFP or EGFP-ILK-E359K proteins are shown. Fewer HTR8-SVneo cells migrated into the wound when they were transiently transfected with dominant negative pEGFP-ILK-E359K compared to cells transiently transfected with EGFP empty vector. Vertical bars indicate the position of the wound in the monolayer. The experiments were done in triplicate. Scale bars, 100 μm.

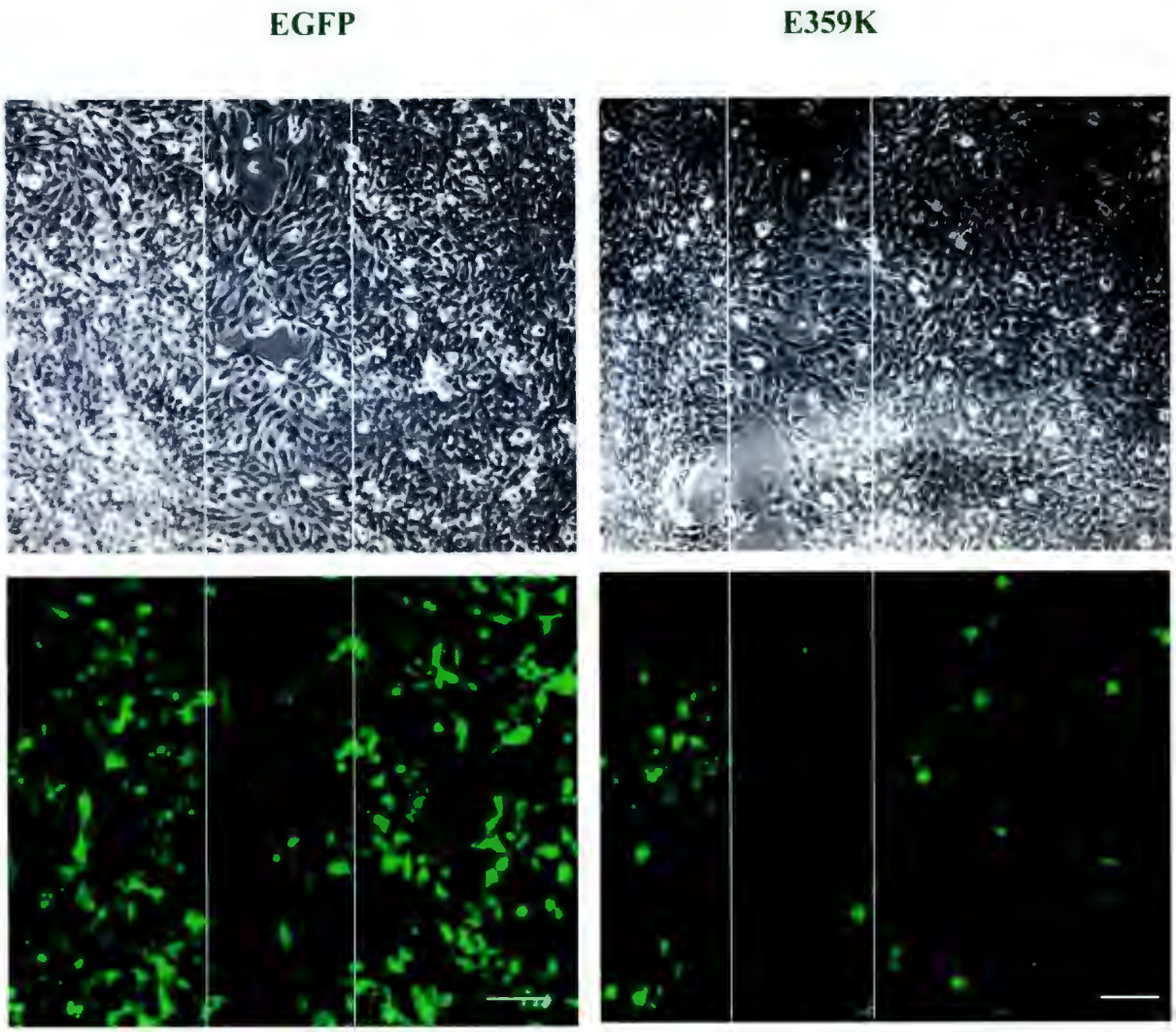
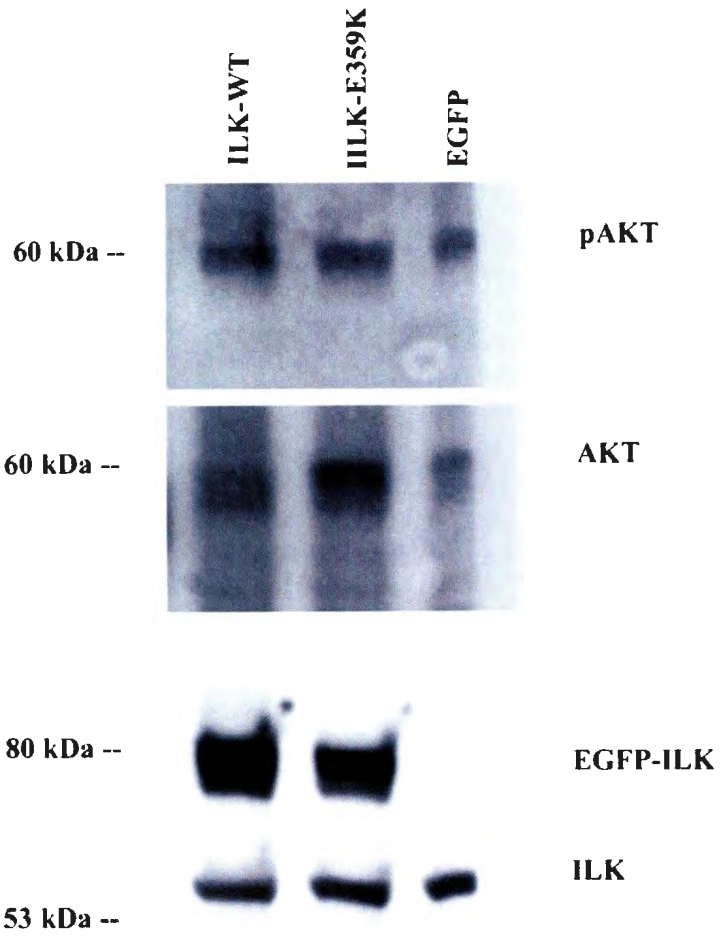

EGFP
E359K

Figure 3.22 pAKT expression in HTR8-SVneo cells transiently transfected with ILK constructs. This representative blot shows that AKT is similarly phosphorylated in cells transfected with dominant negative pEGFP-ILK-E359K (ILK-E395K) when compared to wild type pEGFP-ILK-WT (ILK-WT) and pEGFP (EGFP) transfected cells. ILK expression is comparable in the three groups of cells.


ILK-WT
IILK-E359K
EGFP
60 kDa --
pAKT
60 kDa --
AKT
80 kDa --
EGFP-ILK
ILK
53 kDa --

migration by ILK, HTR8-SVneo cells were transiently transfected with dominant negative ILK-E359K, EGFP empty vector and ILK-WT and stained with anti-ILK and anti-EGFP antibodies to determine if the EGFP-fusion proteins were localized to FAs. The cells transfected with ILK-WT formed evident FAs with a high number of them colocalized with endogenous ILK at the plasma membrane (Fig 3.23 centre panels) while the cells transfected with the dominant negative ILK-E359K showed a homogeneous staining similar to the cells transfected with the pEGFP empty vector (Fig 3.23 top and lower panels).

To further determine if the organization of the cytoskeleton was affected by the disruption of ILK function, cells transiently transfected with the different ILK containing expression plasmids were stained with rhodamine phalloidin. Rhodamine phalloidin is a fluorescent derivative of a toxin produced by the mushroom *Amanita phalloides* that binds to fibrillar actin in the cytoplasm of the cells by intercalating between the actin subunits (Wulf et al. 1979). The staining of the fibrillar actin in the cells transiently transfected with EGFP empty vector showed nice elongated actin filaments and so did the cells transfected with EGFP-ILK-WT (Fig 3.24). In contrast, the expression of dominant negative ILK-E359K altered fibrillar actin structure. This was evident by the short and disorganized fibrillar actin in those cells that transiently expressed the dominant negative form of ILK (Fig 3.24).

Figure 3.23 ILK and focal adhesions. HTR8-SVneo cells were transiently transfected with EGFP-empty vector, dominant negative pEGFP-ILK-E359K, and pEGFP-ILK-WT. After 48 h post transfection the cells were fixed and double immunostained with antisera specific for ILK (red) and EGFP (green). In addition, the panels on the right show DAPI stained nuclei (blue). The EGFP-ILK-WT is able to localize to focal adhesions (white arrows) in contrast to the EGFP-ILK-E359K. Scale bar, 50 μm.

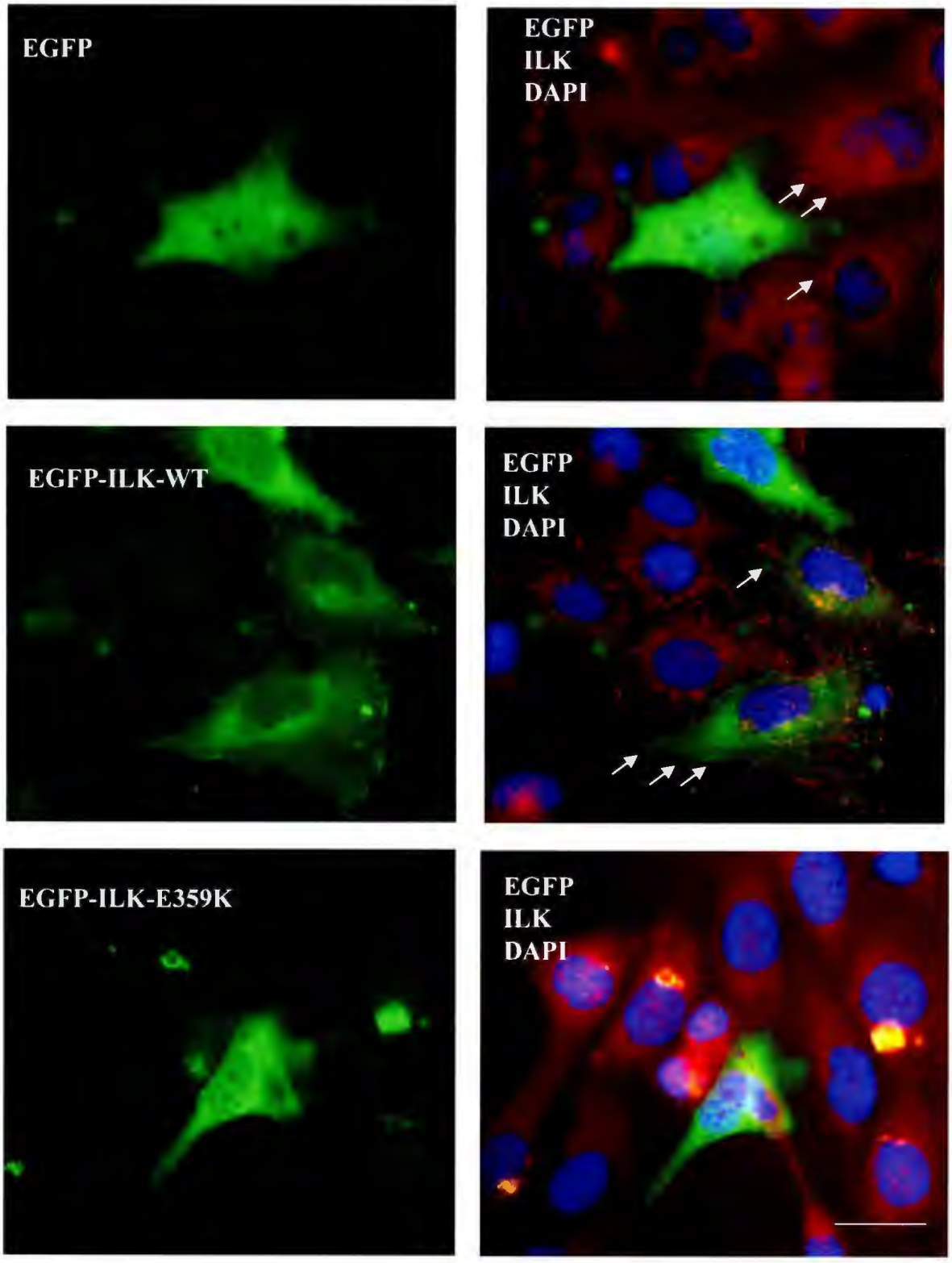
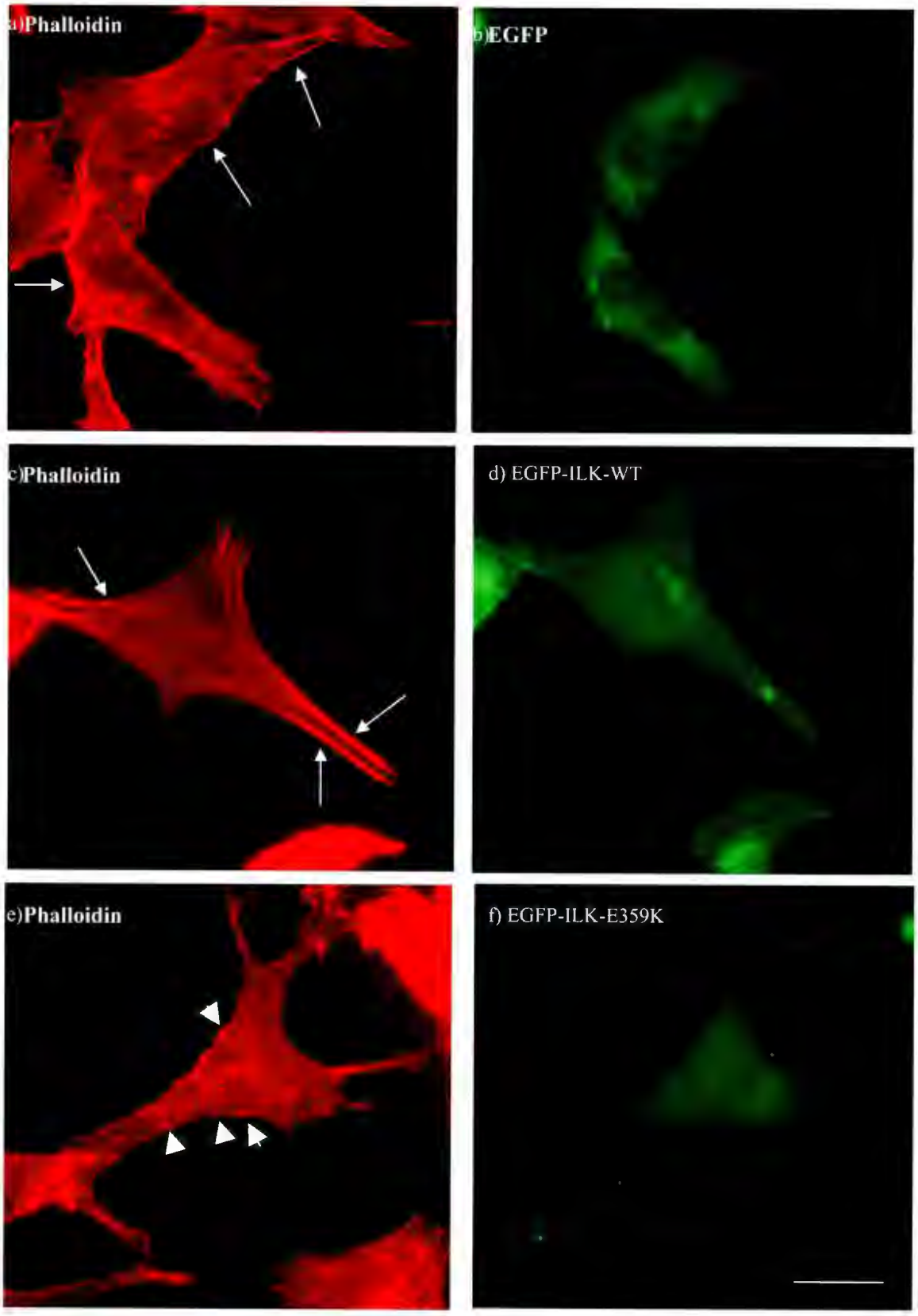

EGFP
EGFP
ILK
DAPI
EGFP-ILK-WT
EGFP
ILK
DAPI
EGFP-ILK-E359K
EGFP
ILK
DAPI

Figure 3.24 ILK and actin organization. The staining of the fibrillar actin in HTR8-SVneo cells transiently transfected with EGFP empty vector (a, b) showed nice elongated fibrillar actin (long arrows) and so did the cells transfected with EGFP-ILK-WT (c, d). In contrast, the cells transiently transfected with dominant negative ILK-E359K displayed short and disorganized fibrillar actin (arrowheads) (e, f). Scale bar, 25 μm.


a)Phalloidin
b)EGFP
c)Phalloidin
d) EGFP-ILK-WT
e)Phalloidin
f) EGFP-ILK-E359K

CHAPTER 4

ILK IS EXPRESSED IN THE MOUSE PLACENTA AND REGULATES CELL INVASION *IN VITRO*.

4.1 Experimental Rationale

Mouse and human placenta have analogous structures that express homologous genes such as *Mash2 (homolog to human HASH2)* (Alders et al. 1997; Janatpour et al. 1999), *Hand1 (homolog to human HAND1)* (Knofler et al. 1998a), and *Gcm1(homolog to human GCM1)* (Janatpour et al. 1999; Nait-Oumesmar et al. 2000) but the expression of *Ilk* in mouse placenta has never been reported. Since the results (see Chapter 3) suggested that ILK protein is involved in regulation of trophoblast migration and the pattern of expression in human placenta showed that ILK is expressed in those cells that are becoming invasive, there is a need to explore ILK expression in the mouse placenta to determine if it is expressed in trophoblast lineages analogous to invasive human trophoblast. For that purpose mouse placentas were collected at different timepoints during gestation, fixed and studied for spatio-temporal expression of ILK by immunohistochemistry. The long term goal is to test if it is feasible to create a conditional knockout for *Ilk* in the invasive mouse placental lineages.

4.2 ILK Expression in the Mouse Placenta

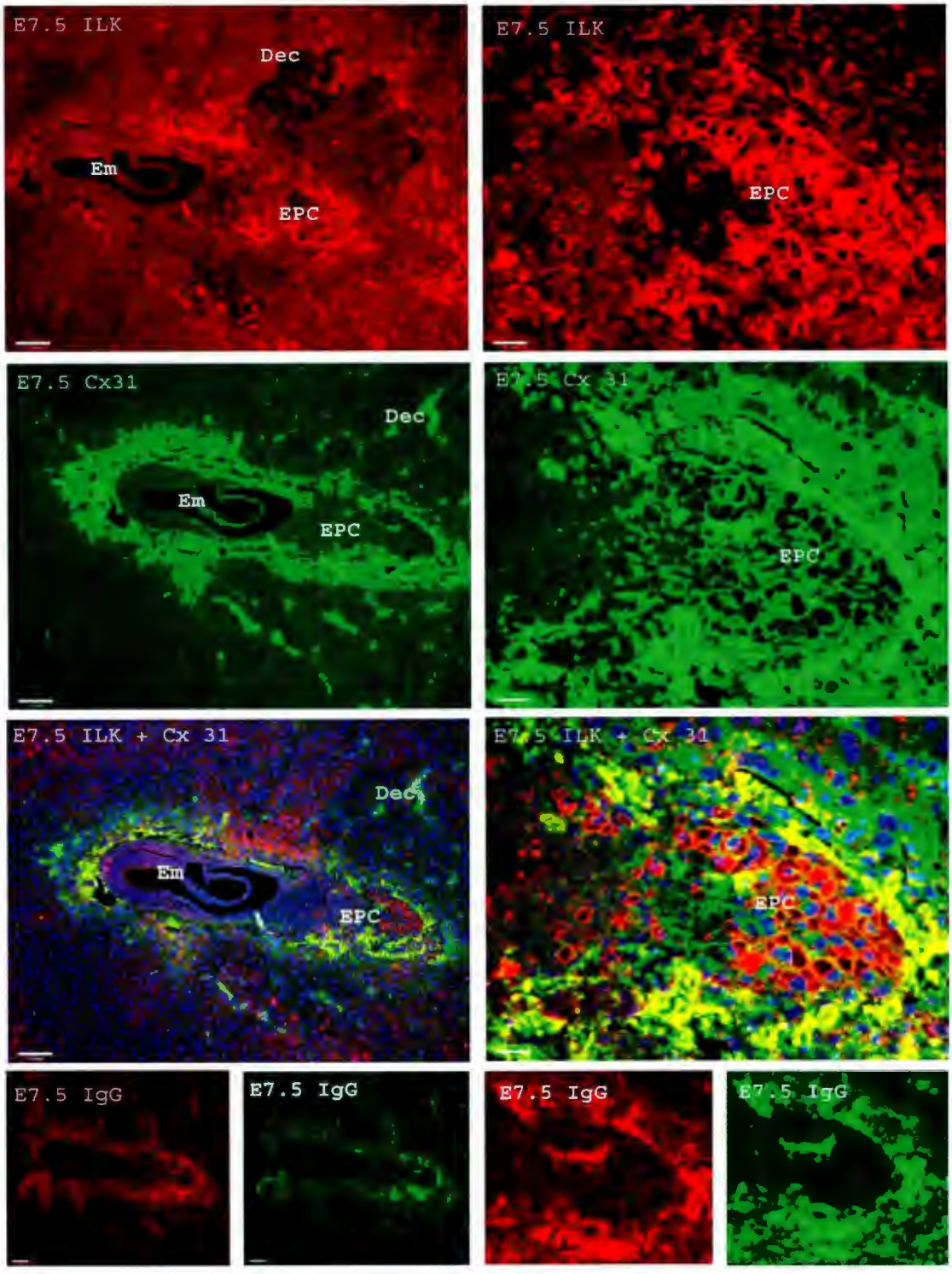
The mouse placenta has a complex structure formed by three layers: the labyrinth consisting of chorionic villi and maternal capillaries, the spongiotrophoblast layer (also called the junctional zone) and the trophoblast giant cell layer surrounded by the maternal decidua (Fig 1.4). To identify the different cell types in each layer immunohistochemistry and immunofluorescence analyses utilizing a combination of several trophoblast layer-specific markers were used. Antisera raised against Connexin (Cx) 26 was used to identify syncytiotrophoblast (Pauken and Lo 1995), Cx 31 to confirm the identification of ectoplacental cone (Grummer et al. 1996), spongiotrophoblast and glycogen cells (GlyC) (Coan et al. 2006); and laminin as a marker of fetal capillaries in the labyrinth (Natale 2006). Periodic Acid-Schiff staining was also used to identify the GlyC in the mouse placenta (Natale 2006).

4.2.1. Developmental Period between E7.5 – E10.5

The precursors of the labyrinth and the spongiotrophoblast layers are found in the ectoplacental cone before E8.5 (Cross et al. 2003a) but the embryo is not completely implanted until E5.5 so samples were collected from E7.5 to E17.5.

ILK was found in the ectoplacental cone at E7.5 (Fig 4.1). This was confirmed by the co-localization with Cx31 (Grummer et al. 1996), a marker for ectoplacental cone at this stage of embryonic development. At E7.5 ILK staining was present

Figure 4.1 ILK is expressed in the ectoplacental cone at E7.5. Shown are representative photographs of ILK and Cx31 immunostaining demonstrating co-localization in the ectoplacental cone (EPC). The figures on the left show a complete embryo with the ectoplacental cone surrounded by decidua. The right panels show a magnified area of the ectoplacental cone with clear ILK or Cx31 immunostaining in the membrane associated areas of the cells. Both the combined pictures also show the nuclei stained with DAPI in blue. Em, embryo; Dec, decidua; EPC, ectoplacental cone. Observed magnification: the panels on the left are at 200X (scale bar, 100 μm), the panels on the right are at 400X (scale bars, 50 μm). The smaller panels at the bottom of the figure are representative images of the IgG controls for mouse and rabbit secondary antibodies.


E7.5 ILK
Dec
Em
EPC
E7.5 ILK
EPC
E7.5 Cx31
Dec
Em
EPC
E7.5 Cx 31
EPC
E7.5 ILK + Cx 31
Dec
Em
EPC
E7.5 ILK + Cx 31
EPC
E7.5 IgG
E7.5 IgG
E7.5 IgG
E7.5 IgG

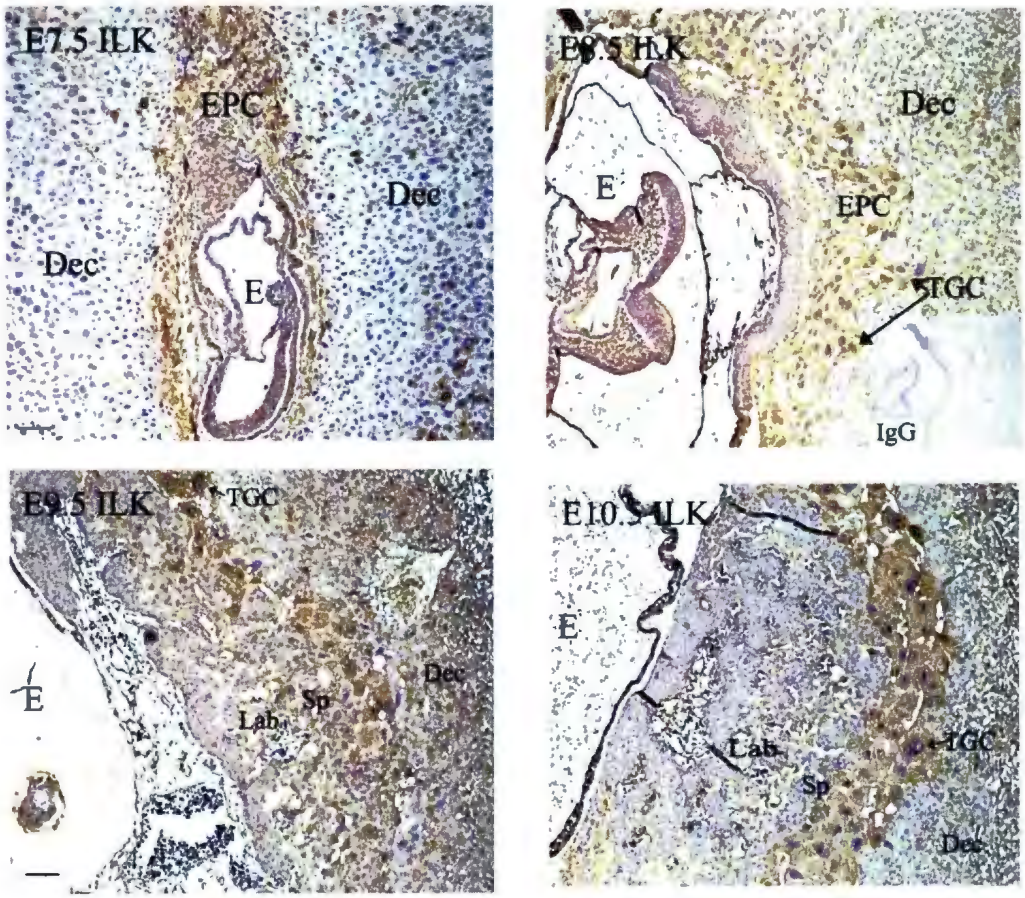
in the ectoplacental cone cells where it seemed to be highly expressed at membrane-associated regions. Ectoplacental cone cells that express Cx31 are invasive (Grummer et al. 1996), suggesting that ILK, which is present in those cells, might play a role in invasion at this stage of development.

From E8.5 to E10.5 the different layers were identified by morphology and localization relative to the embryo. At E8.5 chorionic villi begin to form, immediately after choriallantoic attachment (Cross et al 2003a) and ILK was readily expressed in cells of the embryo as well as in cells of the ectoplacental cone that seemed to be differentiating into labyrinth (Fig 4.2). At E9.5 ILK was localized to the developing labyrinth (Fig 4.2) and also in the trophoblast giant cells (TGCs) that can be identified by their large nuclei at the periphery of the placenta. At E10.5, ILK immunostaining was clearly less intense in the labyrinth and spongiotrophoblast layers compared to the very prominent detection in the cytoplasm and nuclei of TGCs. Only a few cells expressed ILK in the decidua at E7.5. In contrast, at E8.5 and onwards, ILK was expressed in the majority of the decidua cells.

4.2.2 Developmental Period between E12.5 - E17.5

Unlike the human placenta, where the definitive structure of the placenta becomes apparent as early as day 21 of gestation, the mouse placenta does not achieve its definitive structure until halfway through gestation. By E11.5 the labyrinth is completely formed but GlyC do not appear within the spongiotrophoblast layer until about E12.5 (Hemberger and Cross 2001).

Figure 4.2 ILK is expressed early in gestation in the mouse placenta. Shown are representative pictures from embryonic day E7.5 to E10.5 demonstrating ILK immunostaining in the ectoplacental cone (EPC) at E7.5 and E8.5 and in the chorionic plate where the labyrinth starts to develop, at E9.5. At E10.5 the labyrinth, the spongiotrophoblast (Sp) and the trophoblast giant cells (TGC) also show expression of ILK. E, Embryo; EPC, ectoplacental cone; Dec, decidua; Lab, labyrinth. IgG control is shown in the panel insert. Scale bar, 100 μm.


E7.5 ILK
EPC
Dec
Dec
E
E9.5 ILK
Dec
E
EPC
TGC
IgG
E9.5 ILK
TGC
E
Dec
Sp
Lab
E10.5 ILK
E
Lab
TGC
Sp
Dec

From E12.5 to E17.5, ILK was consistently localized to the labyrinth, junctional zone and TGCs (Fig 4.3 and 4.4). ILK was most highly detectable in TGCs, GlyC and labyrinthine trophoblast. ILK was also highly detectable in some spongiotrophoblast and TGC nuclei. In the following figures (Fig 4.5-4.7) each cell type is identified with the proper markers. During these developmental periods in the labyrinth, ILK was present in trophoblast cells and in endothelial cells lining the fetal capillaries of the labyrinth. To verify these initial observations, the expression of laminin, a marker of fetal capillary endothelial cell basement membranes (Coan et al. 2006), and the gap junction protein Cx 26 that is expressed in syncytiotrophoblast (Pauken and Lo 1995) were studied at E12.5 and E15.5 by immunofluorescence analysis (Fig 4.5 and Fig 4.6). ILK was highly expressed in fetal capillary endothelial cells that expressed laminin in their basement membranes (Fig 4.5). The fetal capillaries were also identified on occasion due to the presence of nucleated red blood cells in the lumen of the capillaries. In addition, ILK was expressed in the mouse syncytiotrophoblast layer that was identified by the expression of Cx 26 (Fig 4.6). At the junctional zone, between E12.5 and E17.5, ILK was expressed in the spongiotrophoblast and GlyC that are analogous to the EVT cells of the human placenta (Fig 4.4). To confirm in which cells of the junctional zone ILK was expressed, spongiotrophoblast and GlyC cells were identified by comparing tissue sections immunostained for ILK with adjacent tissue sections stained with Periodic Acid-Schiff which detects glycogen stored in the GlyC (Fig. 4.7 top two panels). Furthermore, ILK localization to spongiotrophoblast and GlyC was confirmed by performing co-immunolocalization analysis of ILK and Cx 31, a marker of

Figure 4.3 ILK is expressed in the three layers of the mouse placenta from E12.5 to E13.5. Immunohistochemical detection of ILK in mouse placenta showing that ILK is expressed in the labyrinth, the spongiotrophoblast layer and the trophoblast giant cells at all of the time points studied. Lab, labyrinth; Sp, spongiotrophoblast; GlyC, Glycogen cells; TGC, trophoblast giant cells, IgG, isotype matched IgG control from the same species as the primary antiserum used. IgG controls are shown in the panel inserts. Scale bars: 50 μm.

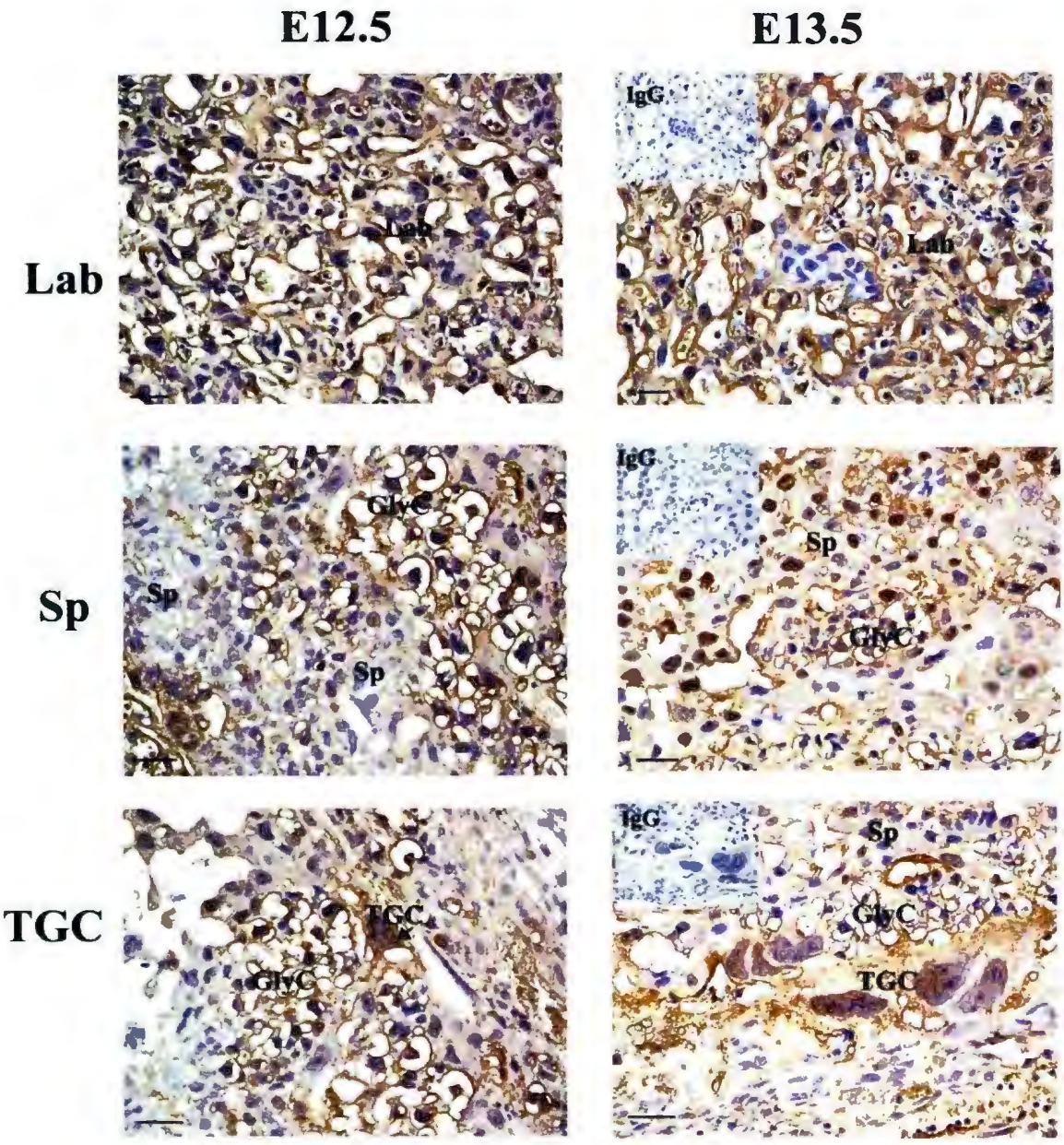

E12.5
E13.5
Lab
Sp
TGC
IgG
Lab
GlyC
Sp
TGC

Figure 4.4 ILK is expressed in the three layers of the mouse placenta from E15.5 to E17.5. Immunohistochemical detection of ILK in mouse placenta showing that ILK is expressed in the labyrinth, the spongiotrophoblast layer and the trophoblast giant cells in all the time point studied. Lab, labyrinth; Sp, spongiotrophoblast; GlyC, glycogen cells; TGC, trophoblast giant cells (indicated by arrowheads); IgG, isotype matched IgG control from the same species as the primary antiserum used. IgG controls are shown in the panel inserts. Scale bars, 50 μm.

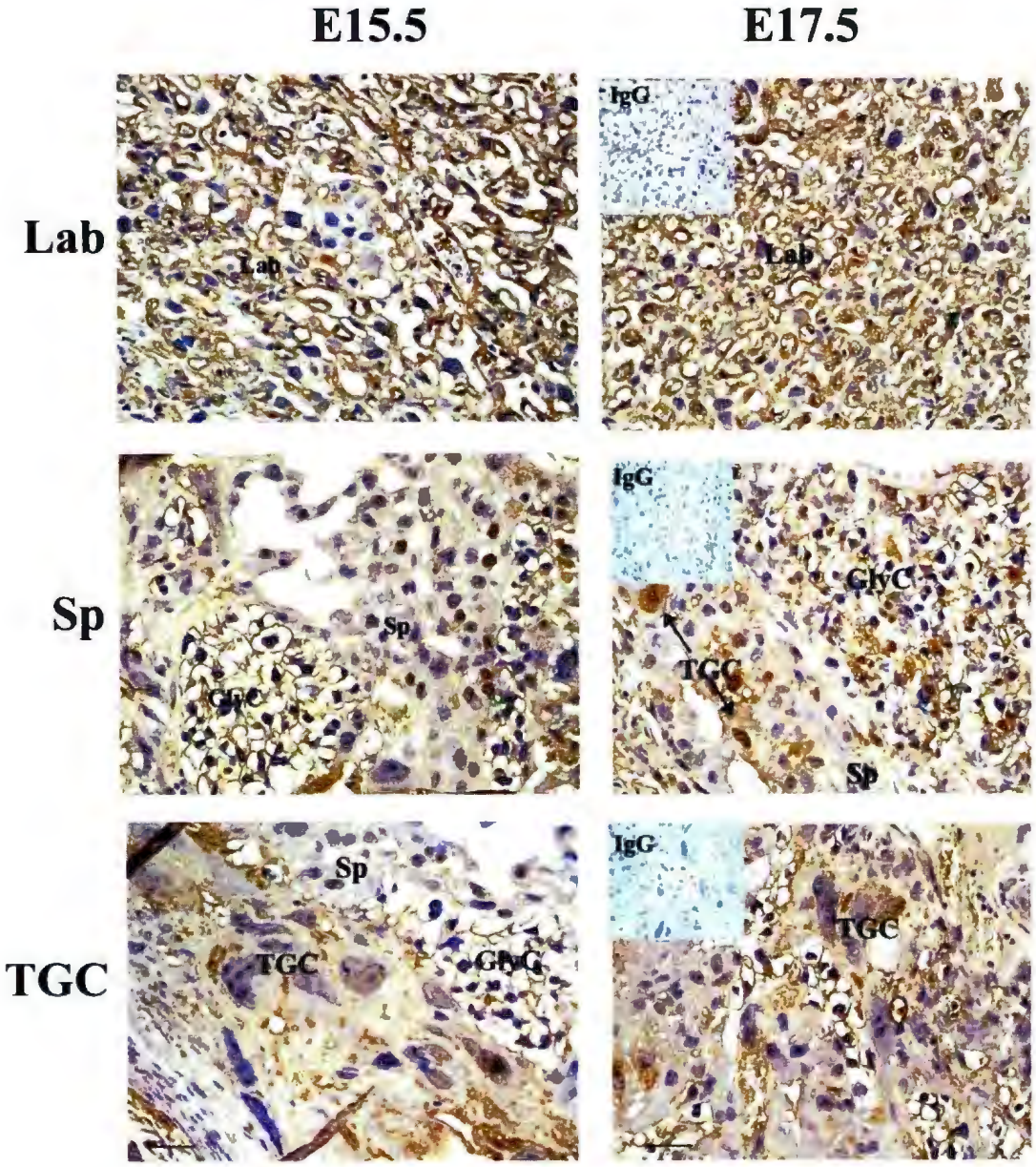
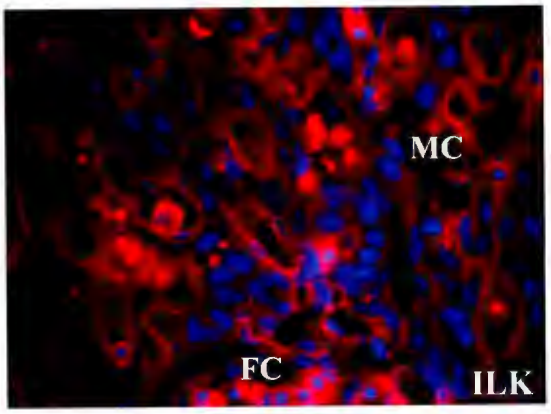
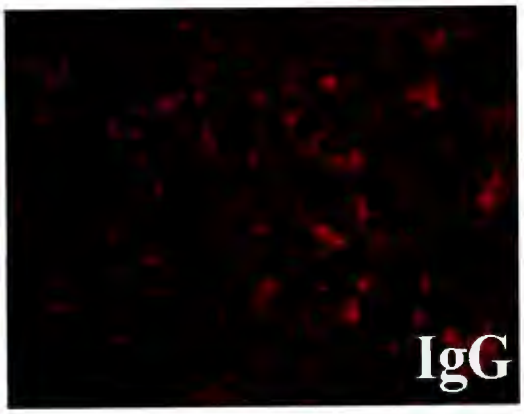
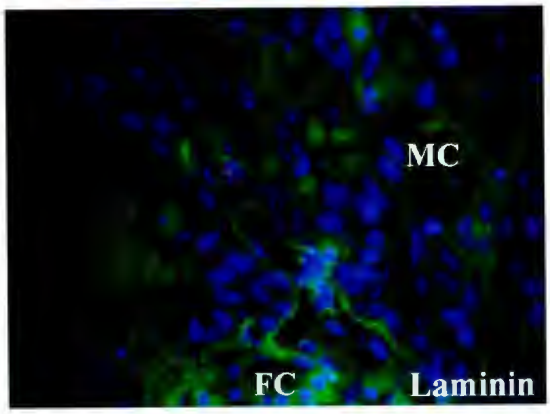
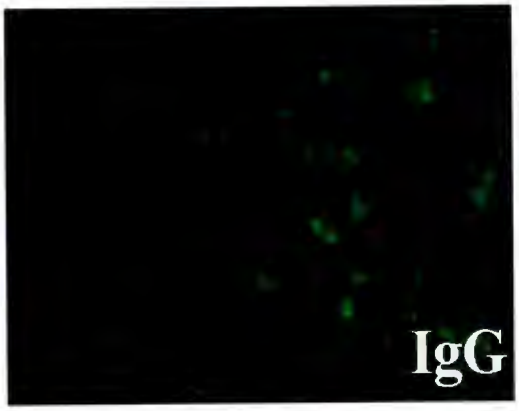

E15.5
E17.5
Lab
IgG
Lab
Lab
Sp
IgG
Sp
GlyC
GlyC
TGC
Sp
TGC
IgG
Sp
TGC
GlyC
TGC

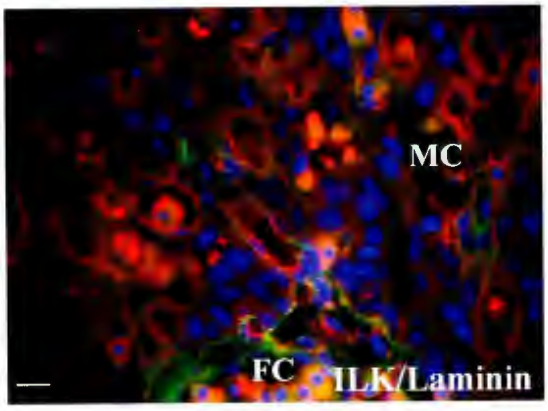
Figure 4.5 ILK is expressed in the mouse placental labyrinth. In this representative image of an E12.5 mouse placenta, ILK (in red) localized adjacent to laminin (in green) that was used here as a marker of the basement membrane of the mouse fetal capillary endothelial cells. Nuclei were stained with DAPI (in blue). The bottom right panel includes a cartoon showing that in the mouse placenta the syncytiotrophoblast is in contact with the fetal endothelial cells. FC, fetal capillary; MC, maternal capillary; IgG, isotype matched IgG control from the same species as the primary antiserum used. Scale bar, 50 μm.

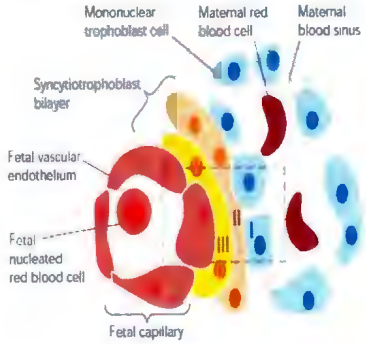












Adapted from Physiology 20:180-193, 2005

Figure 4.6 ILK is expressed in the syncytiotrophoblast. Representative image of E15.5 mouse placenta showing that ILK (in red) is expressed in the syncytiotrophoblast cells. Cx26 (in green) is a marker for syncytiotrophoblast in the mouse placenta. Nuclei were stained with DAPI (in blue). Arrows are pointing to the areas where it is evident that ILK and Cx26 are in the same cells. Cx26, connexin 26; IgG control, isotype matched IgG control from the same species as the primary antiserum used; Scale bars, 50 μm.

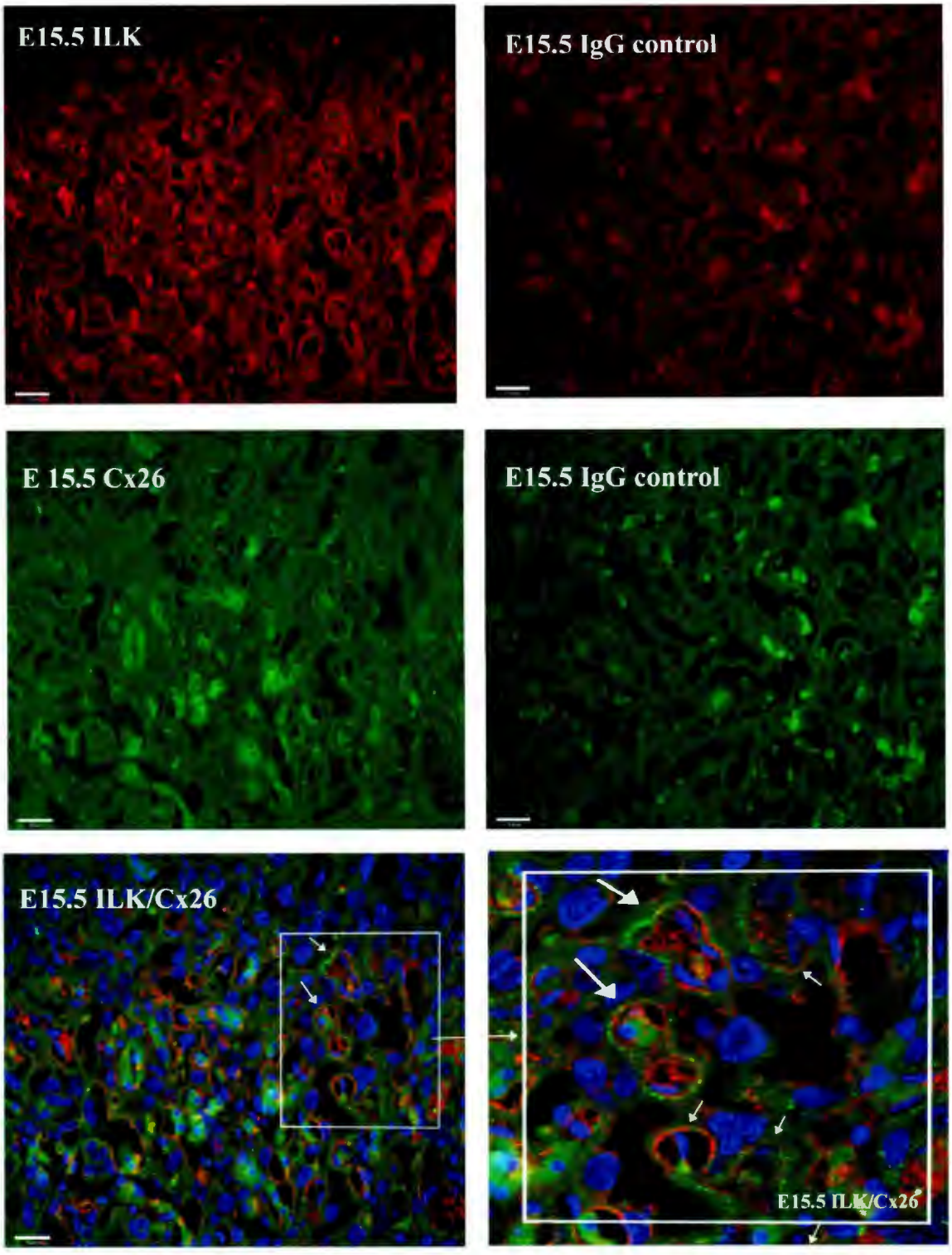
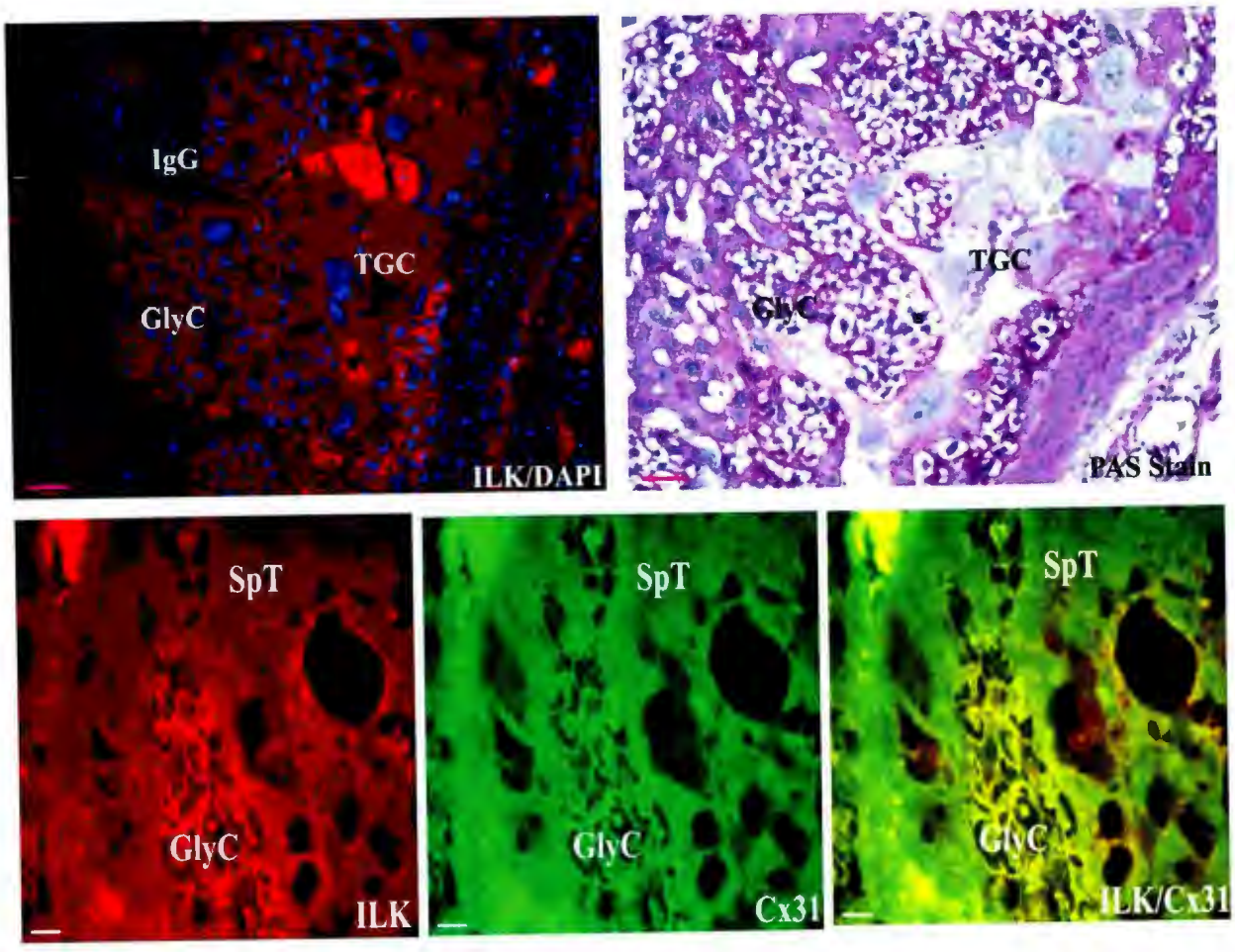

E15.5 ILK
E15.5 IgG control
E 15.5 Cx26
E15.5 IgG control
E15.5 ILK/Cx26
E15.5 ILK/Cx26

Figure 4.7 ILK is expressed in the Glycogen cells of the junctional zone. Top panels: Representative adjacent tissue sections from E 15.5 mouse placenta showing ILK expression and Periodic Acid-Schiff (PAS) staining. ILK is present in the same Glycogen cells that stain with PAS (dark purple). Lower panels: double immunofluorescence of ILK (red) and Cx31 (green) demonstrating that ILK is expressed in the glycogen cells. IgG control is shown in the panel insert. GlyC, glycogen cells; SpT, spongiotrophoblast layer; TGC, trophoblast giant cells. Scale bars, top panels 50 μm; lower panels 25 μm.


IgG
TGC
GlyC
ILK/DAPI
TGC
GlyC
PAS Stain
SpT
GlyC
ILK
SpT
GlyC
Cx31
SpT
GlyC
ILK/Cx31

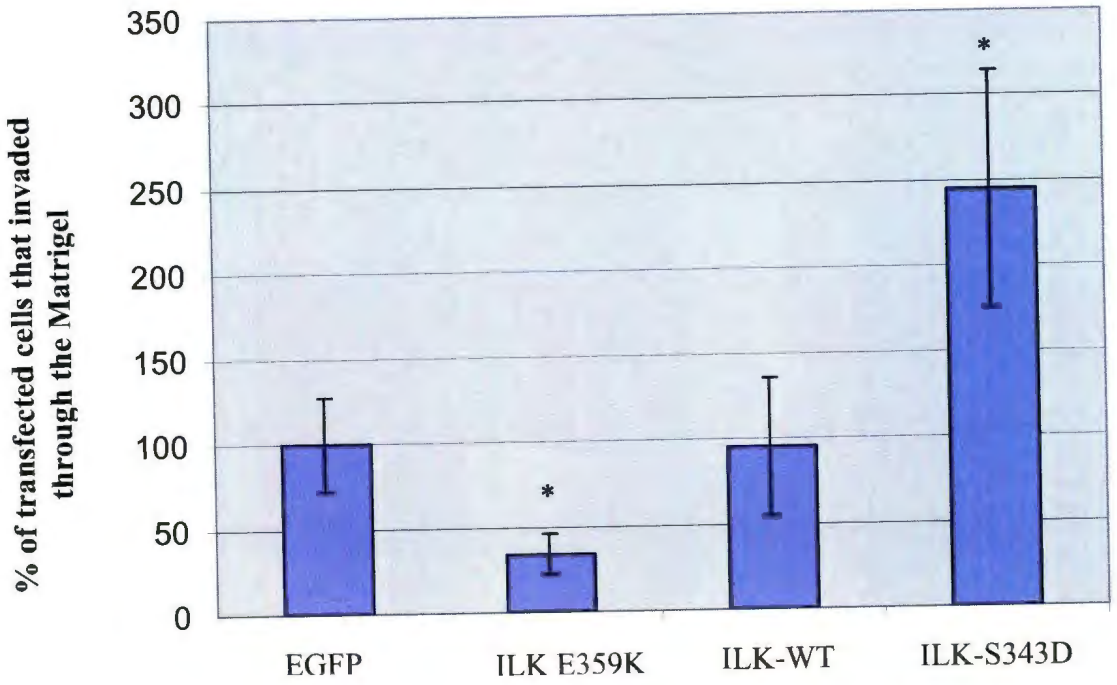
spongiotrophoblast cells and GlyC during this period of pregnancy (Fig 4.7 three bottom panels)

Throughout the gestation times studied, ILK was also found in secondary TGC (Fig. 4.4; right panels, top to bottom). The pattern of staining of the TGC was not equal in all the cells, some expressed ILK mainly in the cytoplasm while others displayed strong expression in the cell nuclei. The TGCs were easily identified by their location in the placenta and their readily visible large nuclei.

4.3 ILK Regulates Trophoblast Invasion *In Vitro*

Since ILK was expressed in human EVT (Fig 3.5), regulated trophoblast migration (Fig 3.16 and 3.17), and was detected in mouse invasive GlyC and TGCs (Fig 4.4), the role of ILK in the process of human trophoblast invasion was studied *in vitro* utilizing the HTR-SVneo trophoblast cell line originally derived from human EVT explants (Graham et al. 1993). Human HTR8-SVneo cells become invasive on Matrigel after incubating them under low serum conditions (Graham et al. 1993, Kilburn et al. 2000). HTR8-SVneo cells transiently expressing dominant negative pEGFP-ILK-E359K were markedly inhibited in their ability to invade through Matrigel (Fig 4.8; $P<0.05$) compared to control cells transfected with an empty pEGFP vector. When HTR8-SVneo cells transiently expressed a constitutively active form of ILK, pEGFP-ILK-S343D, invasion of these cells was significantly increased compared to the control group and pEGFP-ILK-E359K expressing cells ($P<0.05$). No

Figure 4.8 Quantitative analysis of the invasion assays with HTR8-SVneo cells transiently overexpressing EGFP-ILK fusion proteins. The number of EGFP-expressing cells were counted on the underside of each membrane and expressed relative to the total number of transfected cells present in the control. Transient overexpression of pEGFP-ILK-E359K significantly inhibited cell invasion through the Matrigel (ANOVA, $P < 0.05$; Neuman-Keuls post-test) compared to cells overexpressing the control vector (pEGFP) or pEGFP-ILK-WT or pEGFP-ILK-S343D. Transient overexpression of pEGFP-ILK-S343D resulted in an increase in the numbers of cells that invaded the Matrigel compared to control cells (* denotes $P < 0.05$; n=3).

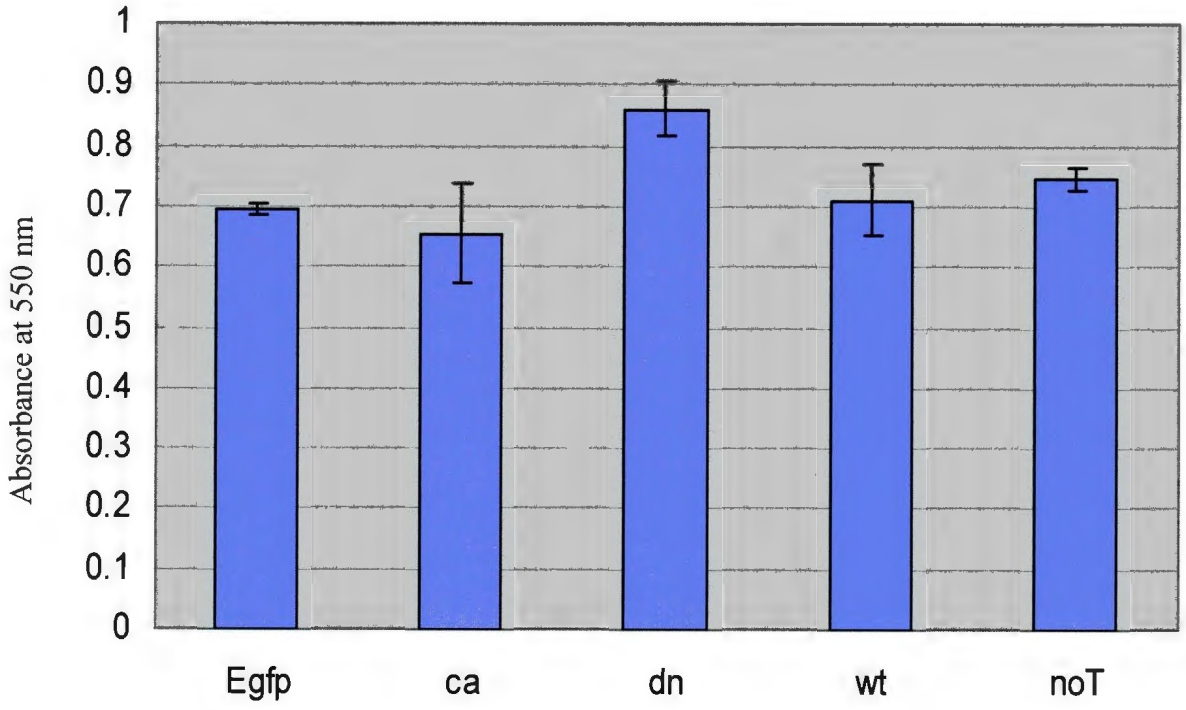


HTR8-SVneo overexpressing different ILK constructs

significant difference in invasive capability was found in cells transfected with pEGFP-ILK-WT compared to pEGFP alone.

In order to study if the proliferation of the cells was affected after the transient transfection with the different pEGFP-ILK plasmids (pEGFP, pEGFP-ILK-WT, pEGFPILK-E359K, pEGFP-ILK-S343D) MTT assays were performed. There were no significant differences in the proliferation of cells transfected with the pEGFP vector control when compared to cells transfected with pEGFP-ILK-WT and pEGFP-ILK-S343D (Fig 4.9). In contrast, cells overexpressing dominant negative pEGFP-ILK-E359K showed increased cell proliferation over a period of 72 h in low serum conditions compared to control cells. ($P< 0.05$; Neuman-Keuls post-test, n=3).

Figure 4.9 MTT proliferation assays. Transient overexpression of constitutively active pEGFP-ILK-S343D (ILK-S343D) or pEGFP-ILK-WT (ILK-WT) in HTR8-SVneo cells did not significantly decrease cell viability/proliferation relative to control HTR8-SVneo cells overexpressing EGFP alone (EGFP) or non-transfected (NoT). In contrast, cells overexpressing dominant negative pEGFP-ILK-E359K (ILK-E359K) showed increased cell proliferation over a period of 72 h in low serum conditions compared to EGFP control cells. (ANOVA, $P < 0.05$; Neuman-Keuls post-test).* denotes significant difference compared to control, $P< 0.05$ (n=3)



HTR8-SVneo overexpressing different ILK constructs

CHAPTER 5

EFFECT OF OXYGEN ON ILK EXPRESSION AND ACTIVITY

5.1 Experimental Rationale

Early human placental development takes place in a low oxygen environment (Rodesch et al. 1992). It is not until the end of the first trimester that there is a significant increase in oxygen tension from about 18 mmHg (3 %) to 60 mmHg (8 %) coinciding with the establishment of the maternal blood flow into the intervillous space (Rodesch et al. 1992). These changes in oxygen tension are critical for normal placental development as trophoblasts proliferate extensively under low oxygen and do not differentiate into invasive cells until the oxygen tension rises above 60 mmHg (Genbacev et al. 1997; Caniggia et al. 2000a). The previous results described in Chapters 3 and 4 indicated that ILK was playing a role in migration and invasion of trophoblast cells. Scandurro et al. (2001) showed, based on computer analysis of the published *ILK* gene sequence (GenBank no. AJ404847), that at least four potential consensus HIF binding sites (RCGTG) called hypoxia responsive elements (HRE) were identified in the *ILK* gene. The HREs found within *ILK* lie 5' to exon 1 as well as between exon 1 and 2 (nucleotides 1042-7, 1107-12, 3526-31, 3533-8). Furthermore, when HepB3, human hepatocellular carcinoma cells were cultured under 1 % oxygen tension, ILK expression was increased (Scandurro et al. 2001; Abboud et al. 2007). Considering these data and the previous results in Chapters 3

and 4, the effect of low oxygen tension on ILK protein and gene expression was studied *in vitro* with HTR8-SVneo trophoblast cells and chorionic villous explant cultures grown at different oxygen tensions.

5.2 *ILK* mRNA Expression Increases Transiently upon Exposure to a Low Oxygen Environment.

To analyze the effect of oxygen tension on ILK gene expression in human trophoblast cells, HTR8-SVneo cells were cultured under low oxygen conditions (3 % O_2) and under normal conditions (20 % O_2). *ILK* mRNA levels were studied by qRT-PCR. First *ILK* mRNA levels at 3 % O_2 were compared to 20 % O_2 after 24 h of incubation of HTR8-SVneo cells in the respective O_2 environment. The level of *ILK* expression was normalized to the expression of the *18S* ribosomal gene that was unaffected by oxygen tension. The amplification efficiencies of the target (*ILK)* and reference gene (*18S*) were similar as calculated by a calibration dilution curve for each gene. There was no significant difference in the expression of *ILK* mRNA in HTR8-SVneo cells grown under low oxygen compared to *ILK* mRNA expression in cells grown at normal oxygen tension over a period of 24 h (Fig 5.1). *ILK* mRNA levels were further determined at different time points of exposure to 3 % O_2 and 20% O_2 tension (30 min, 3 h, 6 h, 12 h and 24 h). There was a transient but significant increase (Fig 5.2) in *ILK* expression in HTR8-SVneo cells cultured under 3% O_2 at 6 h compared to 30 min. *ILK* expression then decreased so by 24 h of incubation the level of *ILK* mRNA was already lower than the corresponding level measured at 20 % O_2.

Figure 5.1 ILK mRNA levels in HTR8-SVneo cells cultured at 3 % vs 20 % O_2. Comparable levels of *ILK* mRNA are detected at 3 % and 20 % O_2 tensions. Cells were cultured under the two oxygen conditions for 24 h and RNA extraction performed as described in Materials and Methods. ILK expression was studied by qRT-PCR utilizing *18S* mRNA as a reference gene. The relative amount of ILK mRNA was calculated using the ΔΔCt method. *ILK* mRNA expression in HTR8-SVneo cells did not show a significant change at 3 % O_2 when compared to cells cultured at 20 % O_2. (ANOVA, Neumann-Keuls post-test, $P< 0.05$; n=3). This graph shows the results of two experiments each with three replicates.

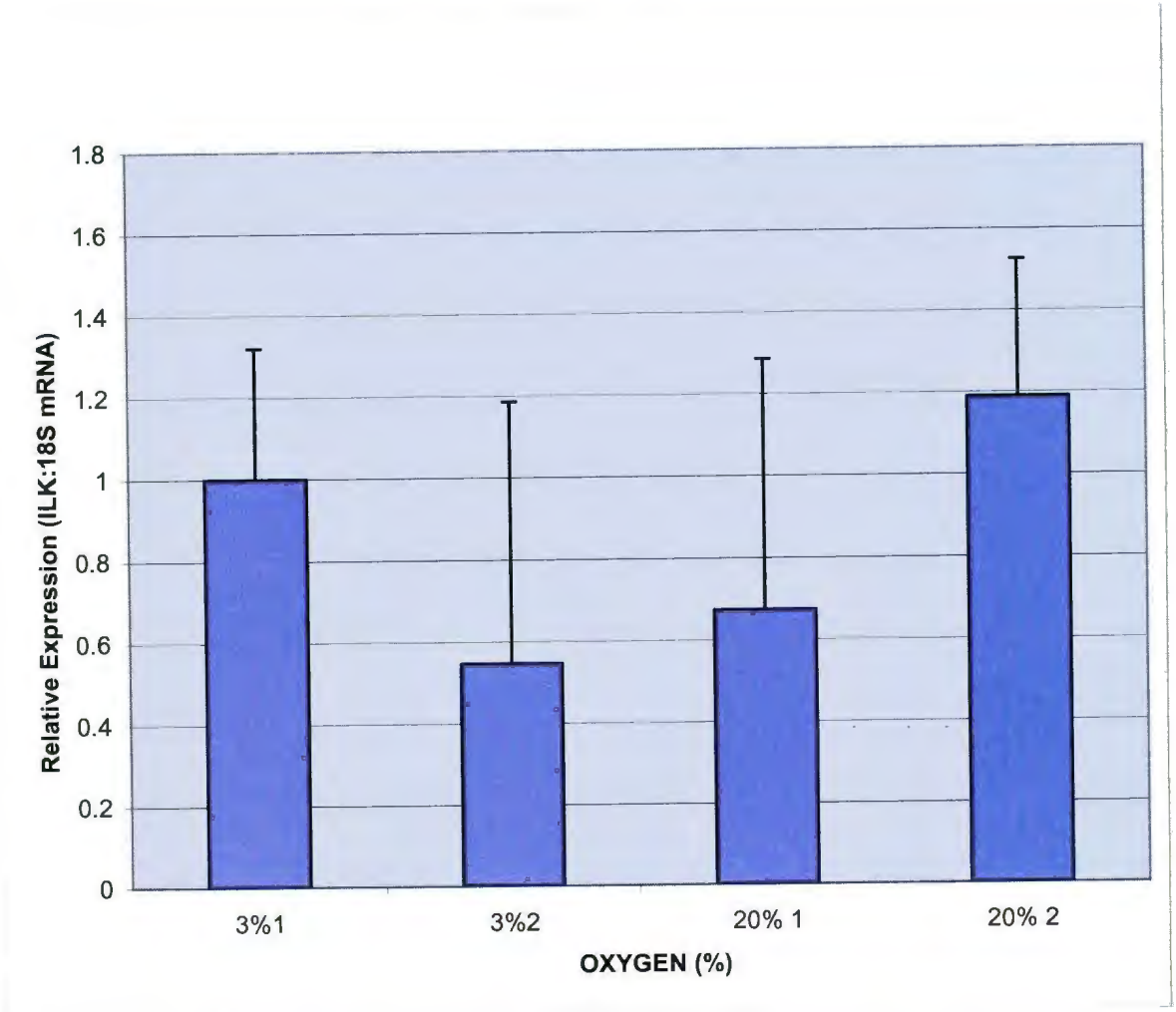
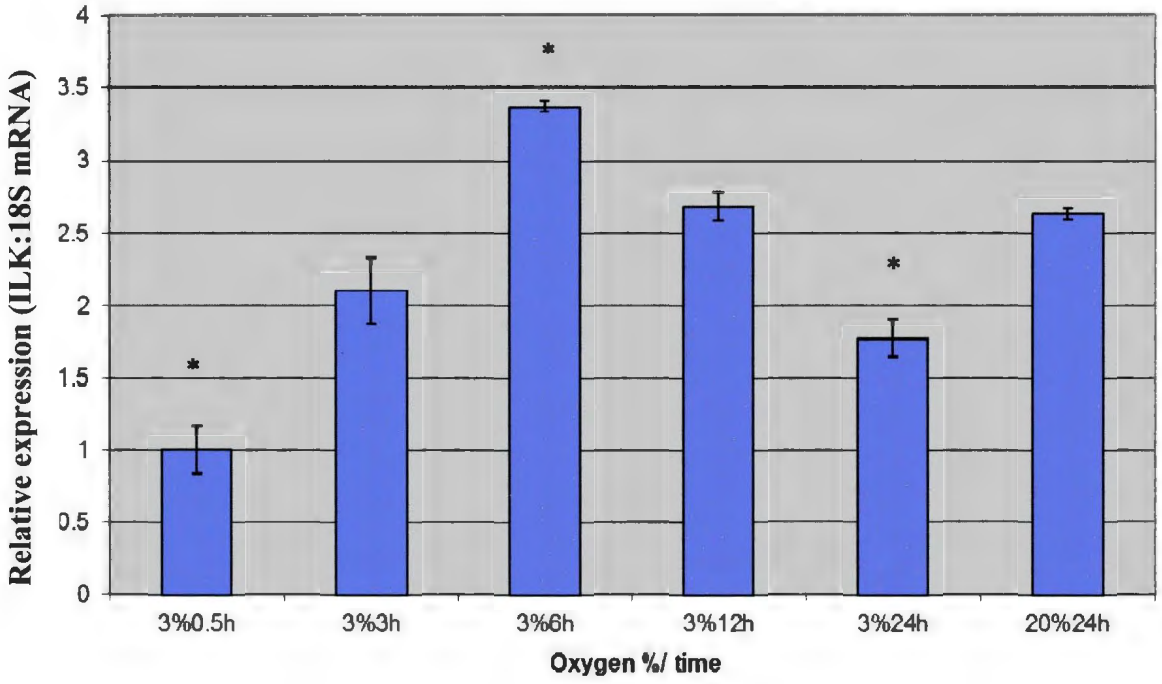

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Expression (ILK:18S mRNA)
3%1
3%2
20% 1
20% 2
OXYGEN (%)

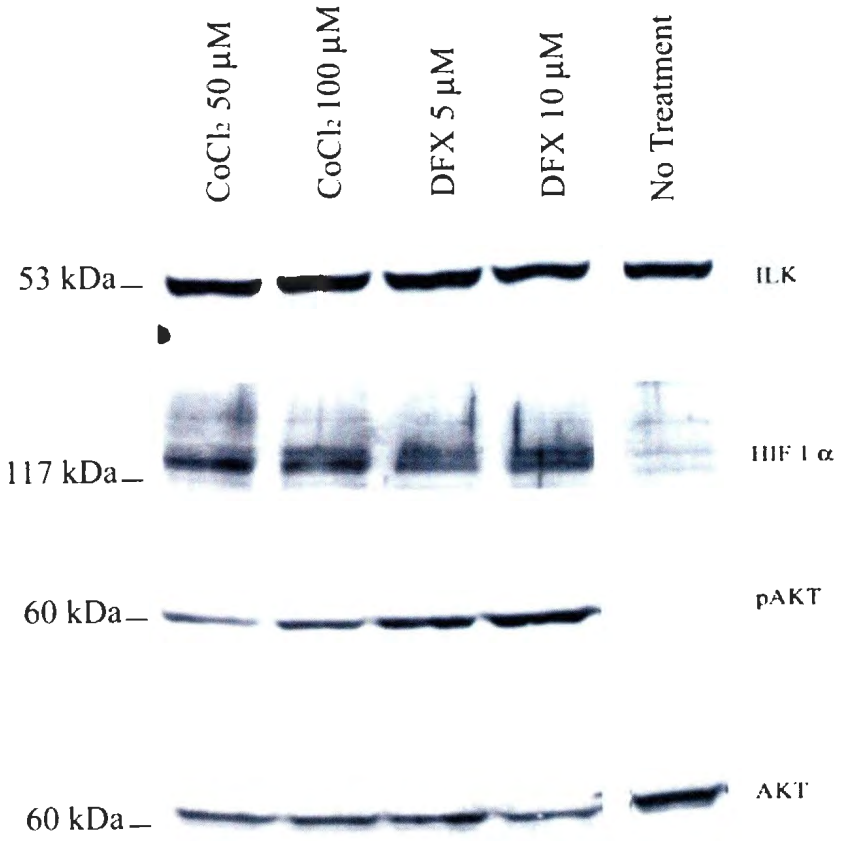
Figure 5.2 ILK mRNA expression in HTR8-SVneo cells increases transiently upon exposure to 3 % O_2. Cells were cultured under 3 % O_2 for 30 min, 3 h, 6 h, 12 h and 24 h and compared with cells cultured for 24 h at 20 % O_2. After each period of time cells were lysed, RNA extracted, and mRNA expression of ILK was studied by qRT-PCR. There was a transient but significant increase in *ILK* expression in HTR8-SVneo cells cultured under 3% O_2 at 6 h compared to 30 min. *18S* rRNA was used as the reference gene and the relative amount of ILK mRNA was calculated using the $\Delta\Delta$Ct method. (*), significantly different form the cells cultured for 24 h at 20 % O_2 (ANOVA, Neumann-Keuls post-test, $P<0.05$; n=3).


Relative expression (ILK:18S mRNA)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
3%0.5h
3%3h
3%6h
3%12h
3%24h
20%24h
Oxygen %/ time

5.3 ILK Protein Expression is Unaltered in Response to a Low Oxygen Environment

The level of ILK protein was studied in HTR8-SVneo cells exposed to hypoxia mimic conditions and compared to normal cell culture conditions. Additionally, ILK protein was measured in cells directly exposed to low oxygen tension. To mimic hypoxic conditions, cobalt chloride ($CoCl_2$, 50 μM or 100 μM) or desferrioxamine (DFX, 5 μM or 10 μM) were added to the regular cell cultivation media. Cobalt can substitute for ferrous iron and becomes incorporated into newly synthesized heme moieties while DFX is an iron chelator (Keberle 1964). In both cases, the heme group is maintained in a deoxy state mimicking a low oxygen environment to the cell, even in the presence of oxygen (Gong et al. 1998; De Marco and Caniggia 2002). Immunoblot analysis was used to study the level of ILK protein in response to the hypoxic conditions. HIF-1α degradation is inhibited in low oxygen tensions so it was used as a marker of exposure to hypoxia (Wang and Semenza 1995; Wang et al. 1995). In normal cells, in the presence of oxygen, HIF-1α is hydroxylated and degraded by the proteasome system so it is virtually undetectable (Salceda and Caro 1997; Ivan et al. 2001). When the HTR8-SVneo cells were exposed to different concentrations of $CoCl_2$ (50 μM or 100 μM) or DFX (5 μM or 10 μM) HIF-1α was detectable while it was not evident in control cells exposed neither to $CoCl_2$ nor DFX. As shown in Fig 5.3 there was no significant difference in the level of

Figure 5.3 ILK protein expression does not change significantly in HTR8-SVneo cells grown under hypoxia mimic conditions. HTR8-SVneo cells were cultured in RPMI 1640 media, containing 10% FBS and 1% Pen /Strep, for 24 h at 37 °C in the presence of either cobalt chloride ($CoCl_2$, 50 or 100 μM) or desferrioxamine (DFX, 5 or 10 μM) to create hypoxia mimic conditions. Cells were then lysed and analyzed for expression of ILK, HIF1α, AKT and pAKT by immunoblot analysis. The immunoblots shown are representative of three experiments.

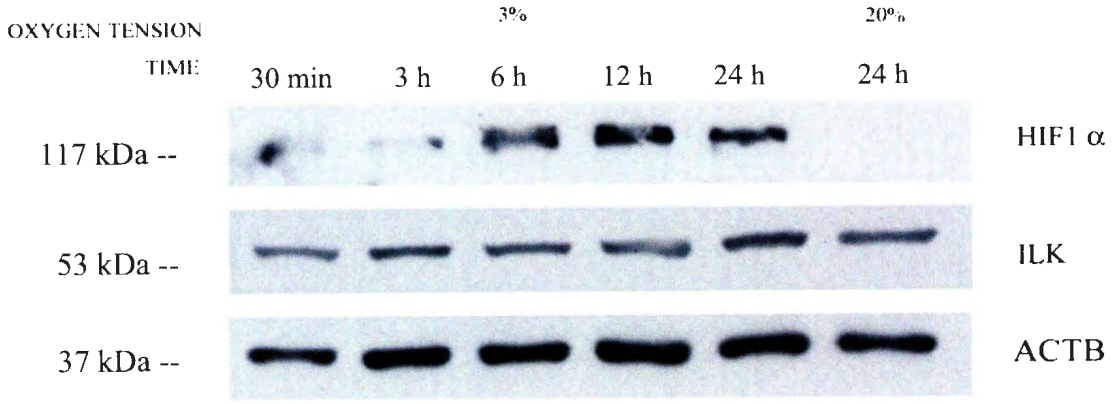

CoCl2 50 µM
CoCl2 100 µM
DFX 5 µM
DFX 10 µM
No Treatment
53 kDa
ILK
117 kDa
HIF 1 α
60 kDa
pAKT
60 kDa
AKT

ILK protein in HTR8-SVneo cells grown under hypoxia mimic conditions compared to normal conditions (20 % O_2). HIF-1α was only detected in cells that had been cultivated in media containing $CoCl_2$ or DFX. Total AKT and pAKT protein levels were analysed in order to test indirectly if ILK kinase activity was affected by the hypoxia mimic conditions, as AKT is a well-known ILK substrate (Delcommenne et al. 1998; Persad et al. 2000). pAKT was detectable in cells exposed to hypoxia mimic conditions but it was not detectable at 20 % O_2 (Fig 5.3) with no significant changes in total AKT levels, suggesting that there was an increase in AKT phosphorylation in HTR8-SVneo cells in response to low O_2.

The effect of hypoxia on ILK protein levels was also studied by cultivating the cells in low O_2 tension conditions instead of mimicking low O_2 with $CoCl_2$ or DFX. For this purpose the cells were placed in a hermetic culture chamber system, within a 37 °C CO_2 tissue culture incubator as described in Materials and Methods. HTR8-SVneo cells were also grown in 20 % O_2 as a control to compare with cells cultivated in the 3 % O_2 environment. The level of HIF-1α was used as an indicator of cells exposed to hypoxic conditions. Cells were incubated in low O_2 conditions (3 % O_2) for 30 min, 3 h, 6 h, 12 h and 24 h and ILK expression was studied by immunoblot analysis. There was no significant change in the level of ILK at any time point of low O_2 exposure when compared to HTR8-SVneo cells cultured at 20 % O_2 for 24 h (Fig 5.4), despite a clear upregulation of HIF-1α expression by 6 h of exposure to 3 % O_2 (Fig 5.4).

As phosphorylation of AKT was increased under hypoxia mimic conditions (Fig 5.3), the importance of ILK signalling in this AKT phosphorylation under a low

Figure 5.4 ILK protein levels do not change in HTR8-SVneo cells in response to a low O_2 environment. HTR8-SVneo cells were exposed to 3 % O_2 for 30 min, 3, 6, 12 and 24 h. A control group of HTR8-SVneo cells were incubated at 20 % O_2 for 24 h. At the end of each period of time the cells were lysed and ILK protein expression was studied by immunoblot analysis. This representative blot of three experiments shows detection of ILK on immunoblots at around 55 kDa. HIF-1α was used as a marker of the hypoxia exposure while β–actin (ACTB) was used as the loading control.

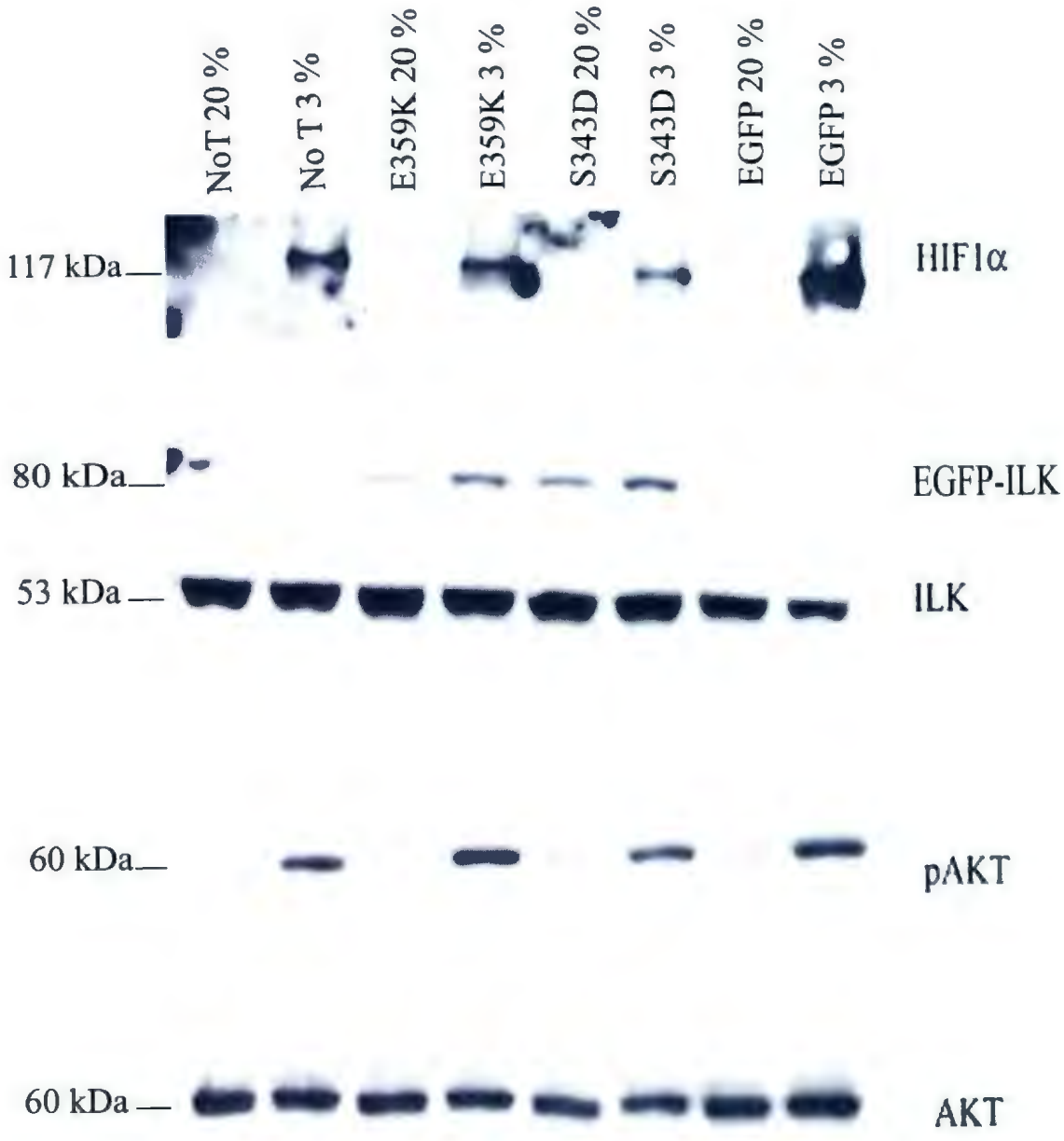

OXYGEN TENSION
3%
20%
TIME
30 min
3 h
6 h
12 h
24 h
24 h
117 kDa --
HIF1 α
53 kDa --
ILK
37 kDa --
ACTB

oxygen environment was determined by transfecting HTR8-SVneo cells with pEGFP, pEGFP-ILK-E359K, or pEGFP-ILK-S343D plasmids. Cells were then incubated at 3 % or at 20 % O_2 for 24 h and the levels of pAKT were analyzed, by immunoblot analysis, in cell lysates collected from each of the conditions. There was no inhibition of AKT phosphorylation in cells transfected with the dominant negative pEGFP-ILK-E359K, nor was there an increase in phosphorylation of AKT detected when cells were transfected with the constitutively active pEGFP-ILK-S343D (Fig 5.5). ILK showed comparable levels of expression in HTR8-SVneo cells cultivated in all the different environmental conditions. HIF-1α detection on immunoblots was included as a positive control for hypoxia exposure and was only present in those cells that were exposed to 3 % O_2.

5.4 ILK Kinase Activity *In Vitro* does not Change Significantly under Low Oxygen Environment

The previous results showed that there was no detectable change in ILK protein expression levels when the cells were cultured under low O_2 conditions. To study if ILK activity was affected by the O_2 environment, HTR8-SVneo cells were cultured under normal and low O_2 tensions in parallel for 24 h, lysed, and then ILK was immunoprecipitated for *in vitro* kinase assays. The first substrate that was tested for this assay was AKT (Cat # 14-279; Upstate). The problem encountered was that the substrate appeared to be undergoing phosphorylation in the IgG-matched control. Several trials to avoid the non-specific phosphorylation, such as adding a preclearing

Figure 5.5 pAKT levels in HTR8-SVneo trophoblast cells increase in 3 % O_2. HTR8-SVneo cells were cultured in either 3 % or 20 % O_2 for 24 h and after cell lysis the extracts were studied by immunoblot analysis with specific antibodies that recognize HIF-1α, ILK, pAKT and AKT. Blots shown are representative of three experiments. No T, non- transfected HTR8-SVneo cells, E359K, HTR8-SVneo transiently transfected with the dominant negative pEGFP-ILK-E359K; S343D, HTR8-SVneo transiently transfected with the constitutively active pEGFP-ILK-S343D; EGFP, HTR8-SVneo transiently transfected with pEGFP; 3 %, 3 % O_2; 20 %, 20 % O_2.

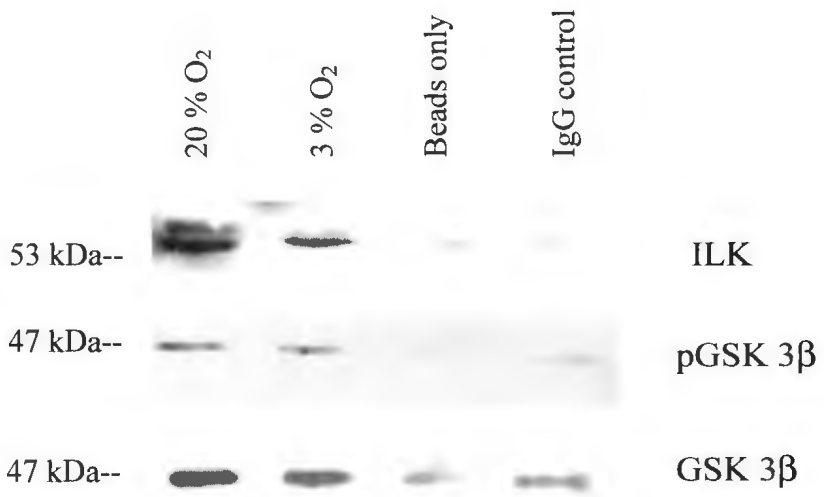

NoT 20 %
No T 3 %
E359K 20 %
E359K 3 %
S343D 20 %
S343D 3 %
EGFP 20 %
EGFP 3 %
117 kDa
HIF1α
80 kDa
EGFP-ILK
53 kDa
ILK
60 kDa
pAKT
60 kDa
AKT

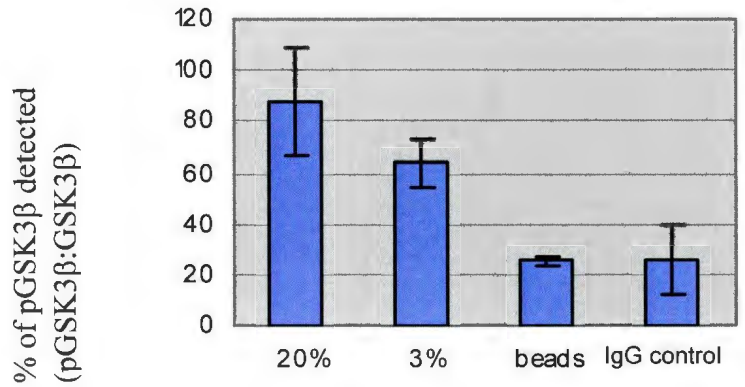
step or increasing the number of washes between the incubations with antibodies failed so the substrate was changed to GSK3β, another well-known substrate of ILK (Delcommenne et al. 1998; Novak and Dedhar 1999; Troussard et al. 1999). As shown in Fig 5.6 there was no significant difference in the activity of ILK in hypoxic conditions compared to 20 % O_2 (Fig 5.6) as suggested by the comparable detection of pGSK3β in both cells exposed to 3 % and 20 % O_2. As expected the detection of pGSK3β in the different controls showed a much lower intensity.

5.5 ILK Expression is Less Detectable in Explants Cultured at Low Oxygen Tensions

A very useful model to study trophoblast differentiation is the explant culture of small samples of human placental chorionic villi. In this model, the chorionic villi are dissected from human placentas and cultured on Matrigel. On the Matrigel, the trophoblasts in the chorionic villi are able to differentiate into invasive cells, mimicking the *in vivo* situation, with the advantage that the architecture of the villi is conserved (Miller et al 2005). To further test if ILK expression was affected by O_2 tension, chorionic villi from week 7 and 11 of gestation were incubated on Matrigel for 6 days at 3 %, 8 % and 20 % O_2 in the laboratory of Dr. Isabella Caniggia at the Samuel Lunenfeld Research Institute (Mt. Sinai Hospital, Toronto, Ontario). After fixation, tissue processing and paraffin embedding (see Chapter 2), explant tissue sections were studied for the expression of ILK by immunofluorescence.

Figure 5.6 ILK kinase activity *in vitro* is not affected by low O_2 tension. HTR8-SVneo cells were cultured at either 3 % or 20 % O_2 for 24 h. After cells were lysed, ILK was immunoprecipitated with a specific antibody. ILK antibody-bead complexes were washed and incubated with GSK3β in the presence of ATP to study ILK kinase activity. The products of this reaction were studied by immunoblot analysis. As a control, beads were incubated with the cell lysate in the absence of ILK antibody. Furthermore, an IgG control was utilized where non-specific mouse IgG, at the same concentration as the primary antiserum, was added to the cell lysate in place of the primary antiserum. Blots shown are representative of three experiments (n=3).


20 % O2
3 % O2
Beads only
IgG control
53 kDa--
ILK
47 kDa--
pGSK 3β
47 kDa--
GSK 3β


% of pGSK3β detected
(pGSK3β:GSK3β)
120
100
80
60
40
20
0
20%
3%
beads
IgG control

The explants developed as expected: at 3 % O_2 there was a lot of proliferation and no evidence of cell column formation as confirmed by the absence of ITGA5 (Fig 5.7). At 8 % and 20 % O_2 the cells proliferated but there was already evidence of trophoblast cell column formation and a proportion of cells in this cell column expressed ITGA5 suggesting that the cells were able to differentiate into less proliferative but more invasive cells (Fig 5.8 and 5.9). ILK was expressed in the villous cytotrophoblasts of the chorionic villi at the three O_2 tensions but it seemed, by the intensity of the signal, that ILK was markedly more detectable in the chorionic villous mesenchyme and in villous cytotrophoblast cells at 8 % and 20 % O_2 compared to detection at 3 % O_2 (Fig 5.10). ILK expression was cytoplasmic with no detectable nuclear staining. In accordance with the results found in human placentas (Chapter 3), the syncytiotrophoblast showed practically no immunostaining with the anti-ILK antibody even at low O_2 tensions.

5.6 ILK Expression in Placentas from Preeclamptic Patients

Although the molecular mechanisms causing preeclampsia are not well understood there is general agreement that a deficient invasion of the trophoblast cells is present invariably in all preeclamptic placentas (Pijnenborg et al. 1980; Pijnenborg 1988; Pijnenborg et al. 1991). Since ILK regulates trophoblast invasion (Chapter 4), it was hypothesized that ILK was reduced in preeclamptic patients who present impaired invasion of the maternal uterus by the trophoblasts.

Placentas from preeclamptic patients (kindly provided by Dr. Isabella Caniggia from The Samuel Lunenfeld Institute, Toronto) (n=6) were analyzed for

Figure 5.7 ILK and ITGA5 expression in chorionic villous explants exposed to a 3 % O_2 environment. Small terminal villi from human placenta were cultured on Matrigel in 3 % O_2 environment for 6 days, then fixed in 4 % PFA, processed and paraffin embedded. The sections were then stained with anti-ILK (in red) and anti-ITGA5 (in green) specific antibodies. ILK and ITGA5 were barely detected in the explants. In the bottom panel, nuclei were stained with DAPI. Scale bar, 50 μm. ITGA5, integrin α5; CT, villous cytotrophoblast, M, mesenchyme of the villous stroma; ST, syncytiotrophoblast.

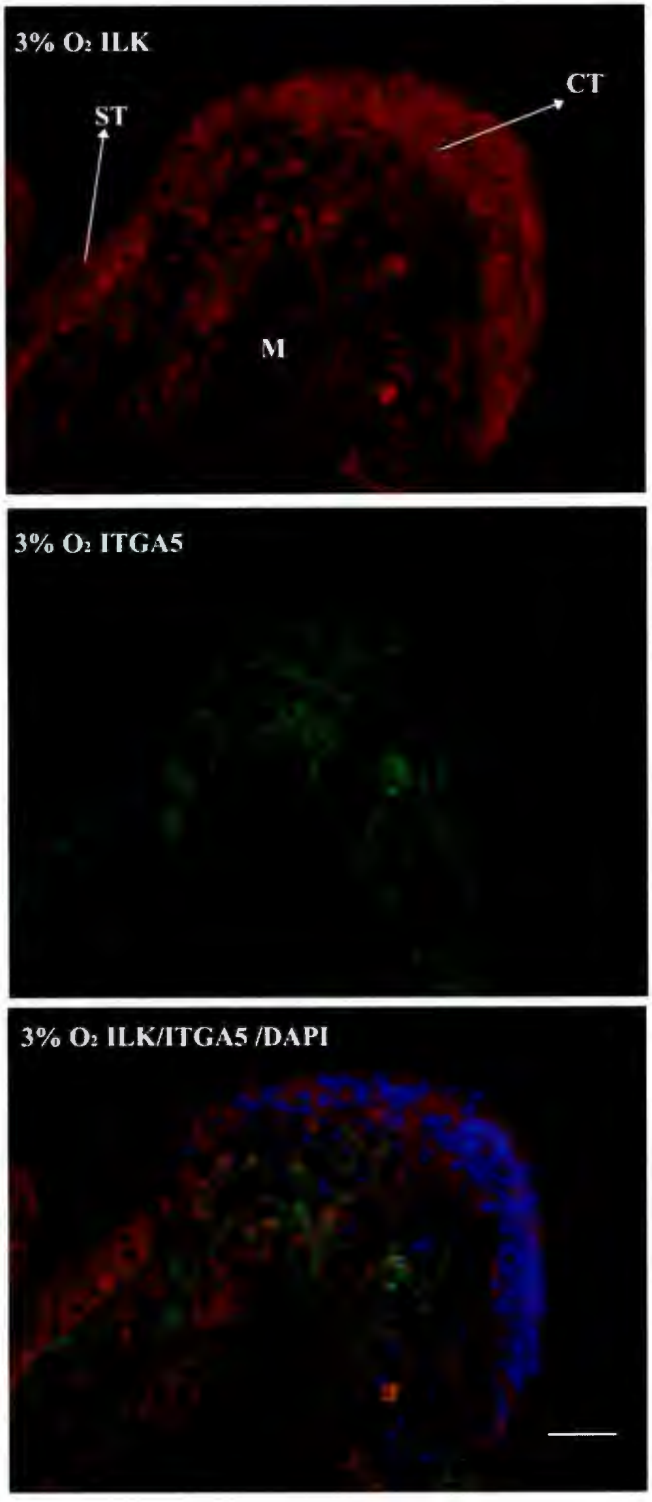

3% O2 ILK
CT
ST
M
3% O2 ITGA5
3% O2 ILK/ITGA5 /DAPI

Figure 5.8 ILK and ITGA5 expression in chorionic villous explants exposed to an 8 % O_2 environment.. Small terminal villi from human placenta were cultured on Matrigel in 8 % O_2 environment for 6 days, then fixed in 4 % PFA, processed, and paraffin embedded. The sections were then stained the with anti-ILK (in red) and anti-ITGA5 antibodies (in green) specific antibodies. In the bottom panel, nuclei were stained with DAPI (in blue). ITGA5, integrin α5; CT, villous cytotrophoblast; M, mesenchyme. Scale bar, 50 μm.

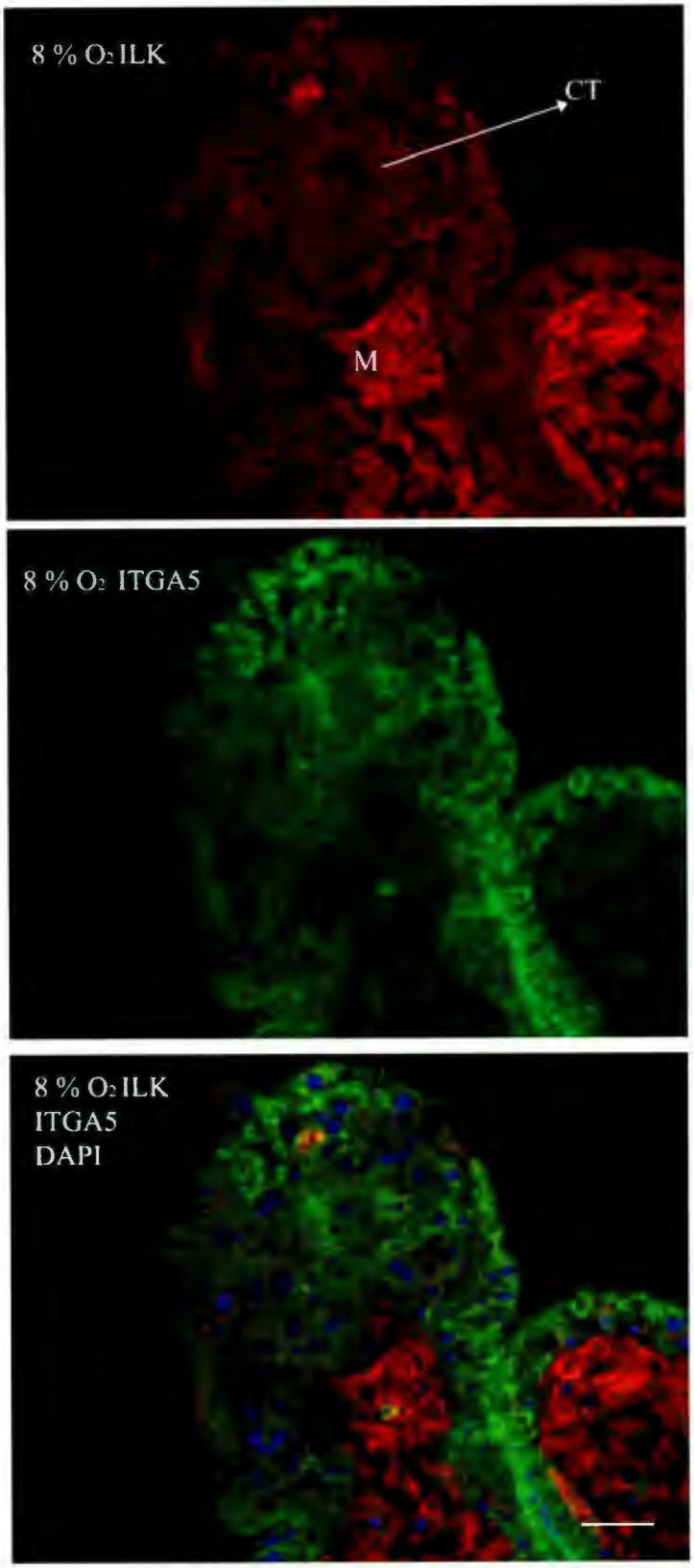
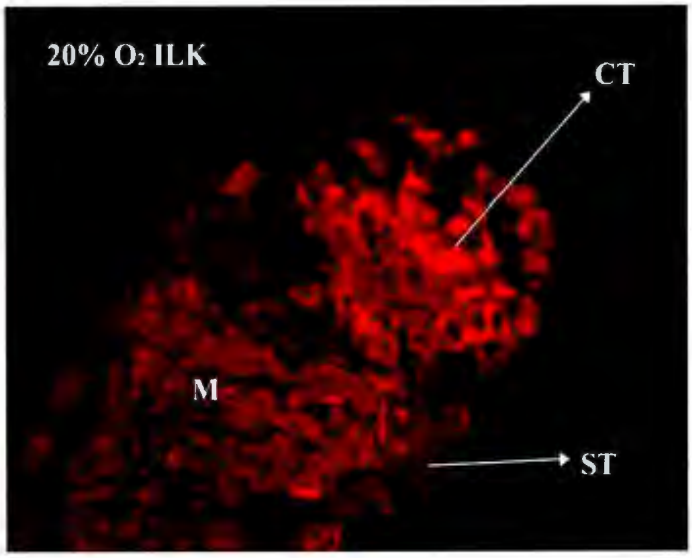
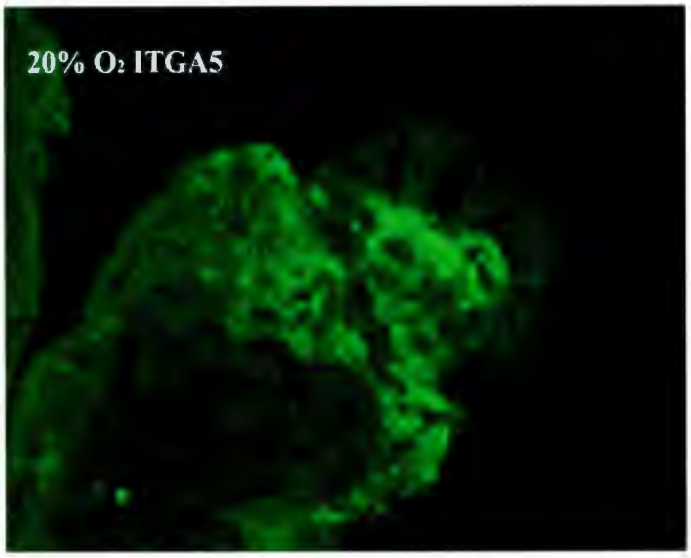

8 % O2 ILK
CT
M
8 % O2 ITGA5
8 % O2 ILK
ITGA5
DAPI

Figure 5.9 ILK and ITGA5 expression in chorionic villous explants exposed to a 20 % O_2 environment. Small terminal villi from human placenta were cultured on Matrigel in a 20 % O_2 environment for 6 days, then fixed in 4 % PFA, processed, and paraffin embedded. The sections were then stained with anti-ILK (in red) and anti-ITGA5 (in green) specific antibodies. In the bottom panel, nuclei were stained with DAPI. ITGA5, integrin α5; CT, villous cytotrophoblast; M, mesenchyme; ST, syncytiotrophoblast. Scale bar, 50 μm


20% O2 ILK
CT
M
ST


20% O2 ITGA5

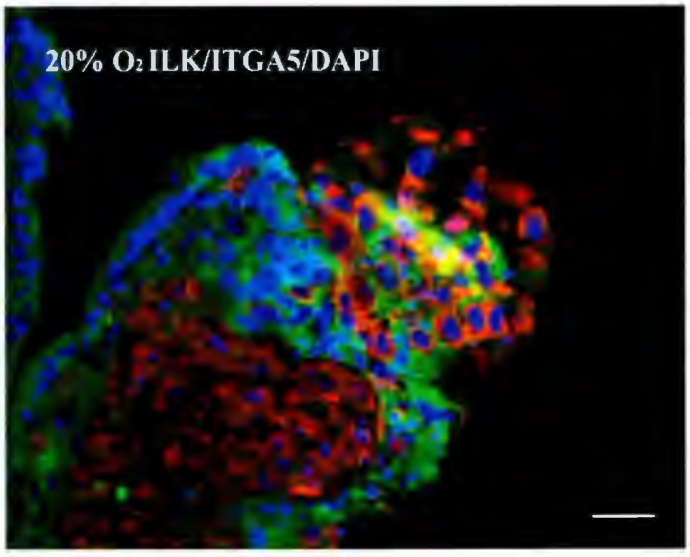
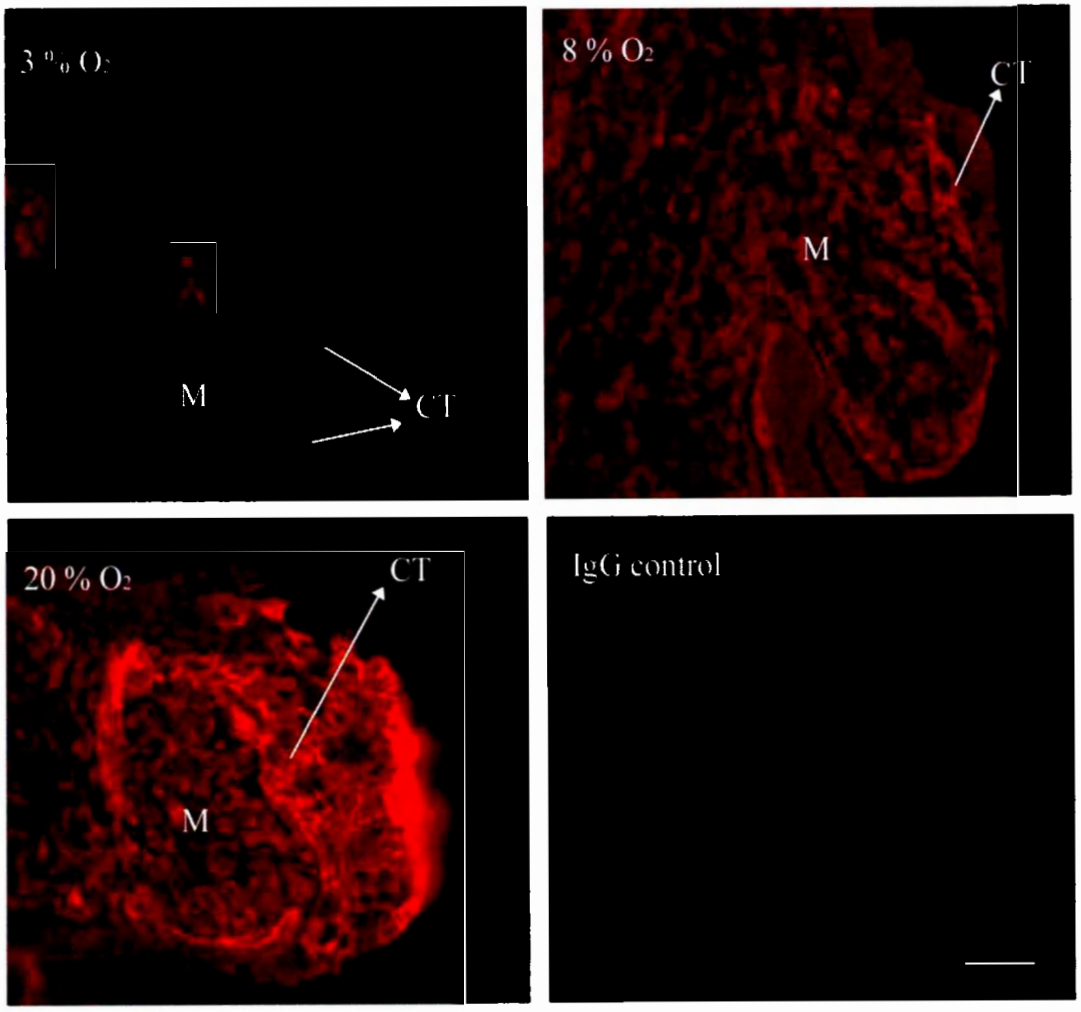

20% O2 ILK/ITGA5/DAPI

Figure 5.10 ILK detection is reduced in chorionic villous explants cultured in a 3 % O_2 environment. Small terminal villi from human placenta were cultured on Matrigel in a 3 %, 8 % and 20 % O_2 environment for 6 days, then fixed in 4 % PFA, processed, and paraffin embedded. Sections of explants were incubated with mouse ILK-specific antibodies. ILK showed a pattern of expression similar to the pattern observed in normal human placenta throughout gestation, when the explant were exposed to 8 % and 20 % O_2. CT, cytotrophoblast; M, mesenchyme; IgG control, non-specific IgG control. Scale bar, 50 μm.


3 % O2
M
CT
8 % O2
CT
M
20 % O2
CT
M
IgG control

ILK expression by immunofluorescence and compared to age-matched controls. Placentas from patients with early preeclampsia onset (from week 20 to week 30, n=3; Fig 5.11) and placentas from patients with late onset of preeclampsia (from week 36 to term, n=3; Fig 5.12) were analysed. The placentas from early preeclampsia onset showed a much lower detection of ILK in endothelial cells of the blood vessels when compared to the aged-matched controls. The tissues from late onset showed a staining pattern in the villous cytotrophoblast and the mesenchyme that was similar to the age-matched controls, but ILK expression still seemed reduced in the endothelial cells of fetal capillaries. In all cases studied, the syncytiotrophoblast showed a staining pattern comparable to the non-specific isotype matched IgG controls suggesting that there was very little detectable ILK in this layer.

Figure 5.11 ILK expression in placentas from patients with early onset preeclampsia. Placentas from patients with early onset preeclampsia (early PE) and age-matched controls (AMC) were fixed, embedded and stained with ILK-specific antibodies. vCT, villous cytotrophoblast; FC, fetal capillaries; ST, syncytiotrophoblast, M, mesenchyme; IgG control, non-specific IgG control.; w, week of gestation. Scale bar 100 μm.

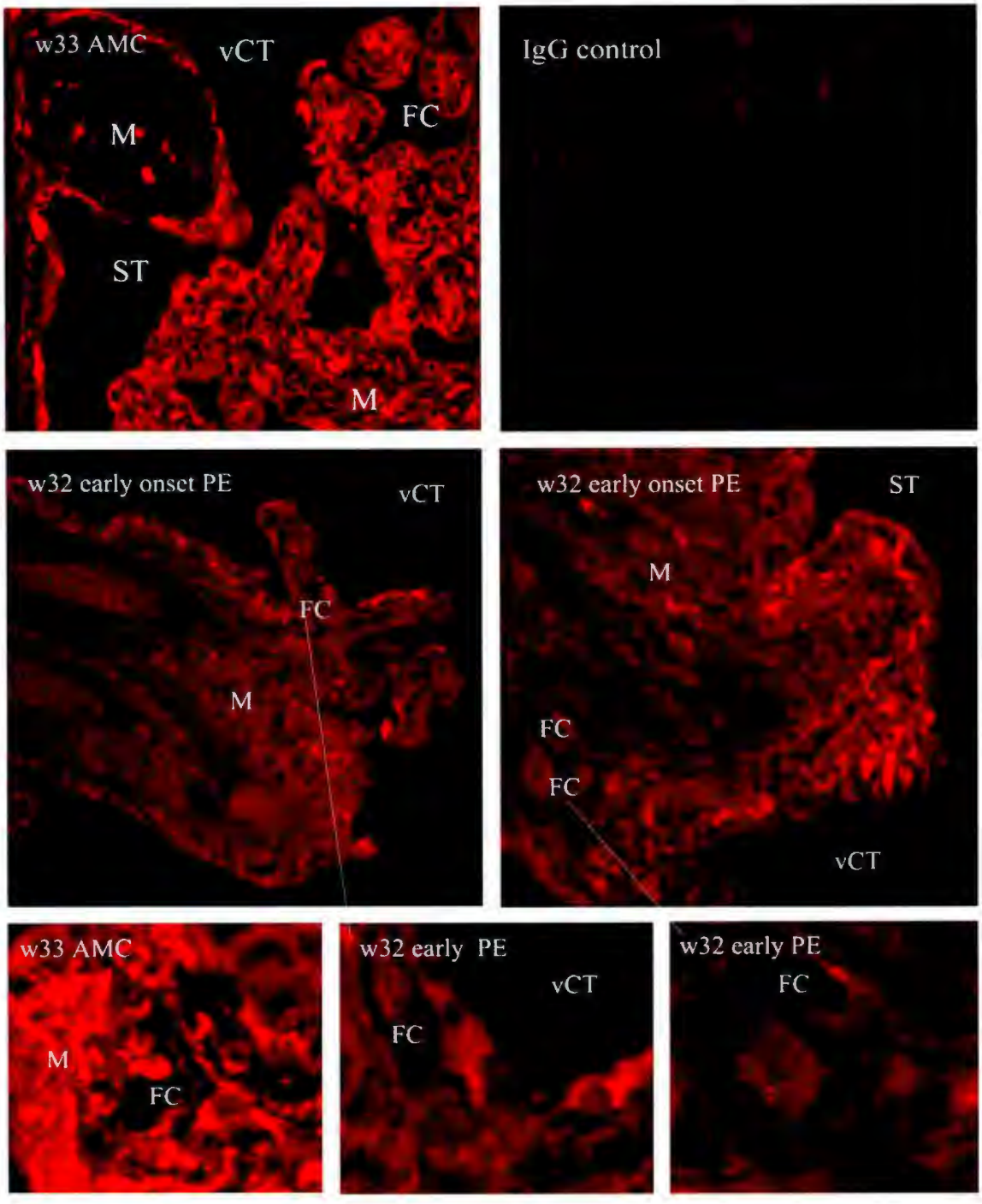
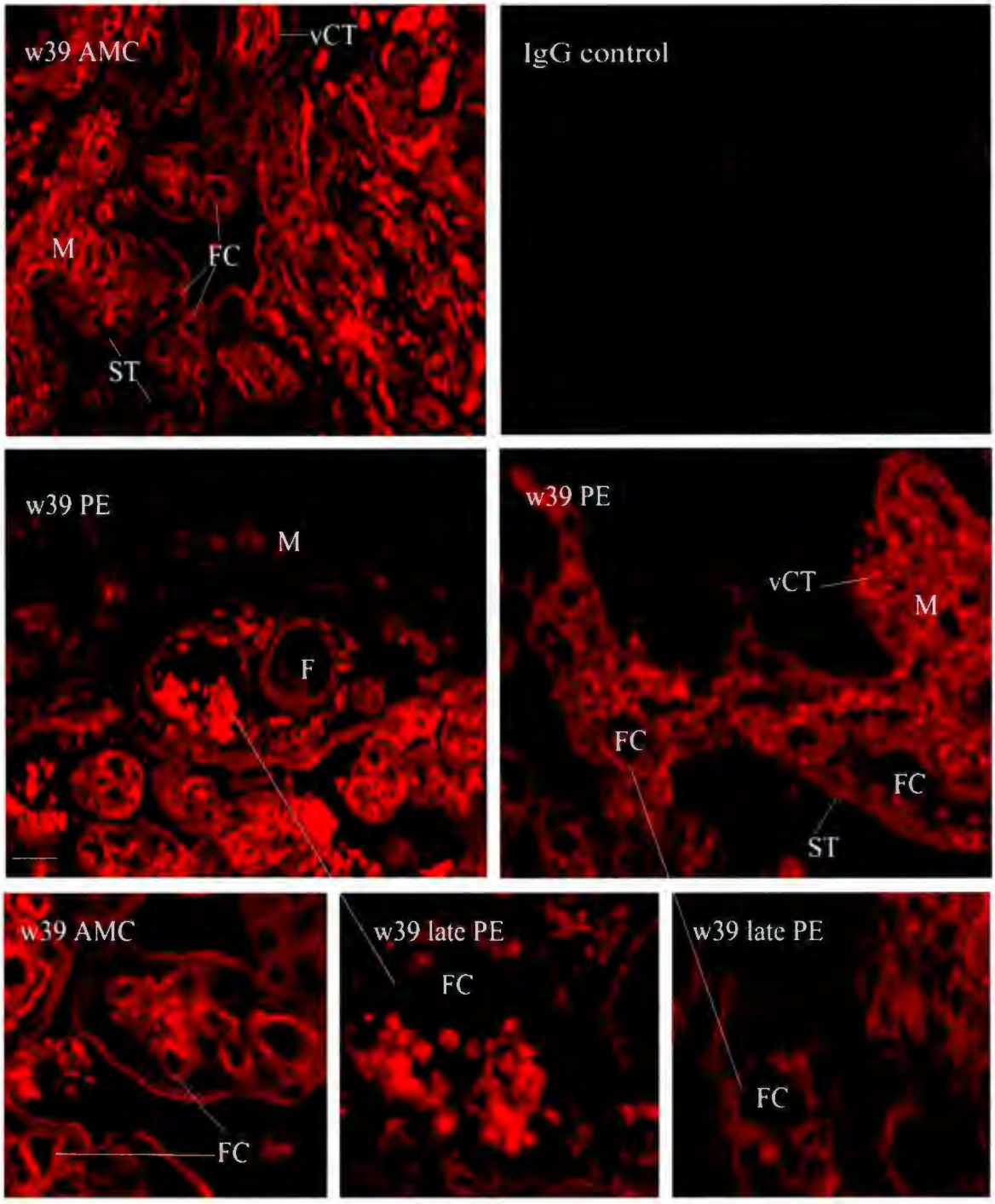

w33 AMC
vCT
M
FC
ST
M
IgG control
w32 early onset PE
vCT
FC
M
w32 early onset PE
ST
M
FC
FC
vCT
w33 AMC
M
FC
w32 early PE
vCT
FC
w32 early PE
FC

Figure 5.12 ILK expression in placentas from patients with late onset preeclampsia. Placentas from patients with late onset preeclampsia (late PE) and age-matched controls (AMC) were fixed, embedded and stained with ILK-specific antibodies. vCT, villous cytotrophoblast; FC, fetal capillaries; ST, syncytiotrophoblast, M, mesenchyme; IgG control, non-specific IgG control.; w, week of gestation. Scale bar, 100 μm.


w39 AMC
vCT
M
FC
ST
IgG control
w39 PE
M
F
w39 PE
vCT
M
FC
FC
ST
w39 AMC
FC
w39 late PE
FC
w39 late PE
FC

CHAPTER 6

DISCUSSION

6.1 ILK is Expressed in Chorionic Trophoblast Cells and Extravillous Trophoblasts of the Human Placenta

Placental development is a complex process that involves the differentiation and invasion of fetal derived cells into the maternal uterus in a finely regulated manner. Trophoblast differentiation during early human placental development involves the two fundamental pathways of fusion or invasion (Zhou et al. 1997; Potgens et al. 2004; Bischof and Irminger-Finger 2005; Pollheimer and Knofler 2005). Some trophoblasts fuse and form the syncytiotrophoblast, others divide and form more trophoblast cells or differentiate losing their proliferative characteristics to become invasive trophoblast cells (Zhou et al. 1997; Potgens et al. 2004; Bischof and Irminger-Finger 2005; Pollheimer and Knofler 2005; Baczyk et al. 2006). Differentiation of trophoblasts into invasive cells is crucial for the proper development of the normal placenta. The trophoblasts must acquire the ability to migrate, reach and modify the maternal arteries to transform them into low resistance vessels. This ensures that enough blood, containing nutrients and oxygen, is supplied to the growing embryo. The trophoblastic cells also require an invasive nature to occupy the decidua and interact closely with uterine cells to coordinately secrete growth factors and hormones crucial for the maintenance of a healthy pregnancy. The

importance of this process becomes evident when the invasion is inadequate and mothers usually develop preeclampsia and the embryo shows signs of growth restriction (Kaufmann et al 2003).

The ubiquitous cytoplasmic serine/threonine kinase named ILK is a regulator of cell proliferation, cell-cell adhesion, migration, and invasion (Hannigan et al. 1996; Wu and Dedhar 2001), processes that are highly pertinent to vCT and EVT developmental pathways. Therefore, the central hypothesis for this thesis was that ILK would be expressed in trophoblast subtypes in human chorionic villi during early placental development and that ILK would regulate trophoblast migration and invasion.

The results from immunofluorescence studies demonstrated that ILK was present in human placenta throughout gestation (see Chapter 3). Interestingly, ILK was detected in the villous cytotrophoblast layer of the chorionic villi and was barely detectable in the syncytiotrophoblast layer (Fig 3.2). Occasionally there was ILK staining on the apical surface of the syncytiotrophoblast. This result may be artifactual because of the presence of microvilli in this layer. It is possible that some residual non-specifically bound primary antisera remained trapped in proximity of these structures despite washes during the experiments, although the presence of ILK in this region cannot be ruled out as many authors have described remainders of intercellular junctions within the syncytiotrophoblast (Carter 1964; Boyd and Hamilton 1966; Burgos and Rodriguez 1966; Metz et al. 1979; Reale et al. 1980; Wang and Schneider 1987). Some investigators have interpreted these junctions as vestiges of a former cellular state of the syncytium or as proof of recent

cytotrophoblastic contribution to the syncytiotrophoblast (Carter 1964; Boyd and Hamilton 1966). In a systematic study of these junctions, Metz et al. (1979) as well as Reale et al. (1980) have described zonulae and maculae occludens that were mostly located in the apical membrane, as well as numerous isolated desmosomes, without contact to any surface membranes (Benirschke and Kaufmann 2000). Furthermore, the Dagnino lab (Vespa et al 2005) showed that ILK is an important modulator of Ca2+-dependent adherens and tight junction formation in epithelial cells so it is possible that ILK might be localized at these "former" cellular junctions but this remains to be directly examined.

The immunostaining in the chorionic floating villi was discontinuous at times as ILK was not detected or detected at a low level in some vCT. These observations suggested that the regulation of ILK expression might be important for cytotrophoblast fusion. These results led to a new project conducted in our laboratory by Trina Butler that showed that ILK facilitates trophoblast fusion (Butler et al 2009). In the cell columns of anchoring villi, both vCT and the proximal tips of EVT cell columns showed ILK expression that colocalized with ITGAV (Fig 3.6). Cruet-Hennequart et al. (2003) have described that ITGAV regulates proliferation of ovarian cancer cells through ILK so, in the human placenta, ILK could be a mediator of proliferation in these specific trophoblast cell populations in response to ITGAV signalling and regulate outside:inside signalling. Distal to the villous tip of EVT cell columns from week 6 to term (Fig 3.7), ILK colocalized with ITGA5. This region of EVT cell columns, marked by detectable ITGA5B1, is known to highly express FAK, paxillin, and MMP 2, and to possess detectable fibronectin, a ligand for ITGA5B1

(Damsky et al. 1992; MacPhee et al. 2001; Bauer et al. 2004). As such, this region of the EVT cell column is characterized as possessing an intermediate, proliferative, migratory phenotype (Damsky et al. 1994). The cytoplasmic tails of beta integrins are mediators of outside-inside signals that come from the ECM (Assoian and Schwartz 2001; Hynes 2002) and interact with the carboxyl terminal end of ILK (Hannigan et al. 1996; Zhang et al. 2004; Chiswell et al. 2008; McDonald et al. 2008). This suggests that integrin-ILK signalling could regulate the differentiation of trophoblasts into migratory cells during placental development. Thus, based on the ILK localization observed, ILK might promote cell cycle progression and trophoblast migration in vCT and EVT, respectively. The overexpression of ILK in mammary epithelial cells promotes anchorage-independent cell cycle progression through upregulation of cyclin D1, and it also suppresses anoikis presumably through the phosphorylation and activation of AKT (Radeva et al. 1997; D'Amico et al 2000). AKT1 is a mediator of cell survival and is also a substrate for ILK, becoming phosphorylated on Ser-473, a critical residue for AKT1 activation (Persad et al. 2001). Notably, pAKT1 was also detectable in placental tissue lysates in all gestational timepoints examined and was immunolocalized to both vCT and EVT cells (Fig. 3.11) suggesting that, in human placenta, AKT might also be a substrate of ILK.

6.2 ILK Regulates Migration of a Trophoblast-Derived Cell Line

To functionally address the role of ILK in the differentiation of EVTs exhibiting an intermediate, migratory phenotype, cell migration assays were conducted with HTR8-SVneo cells, an accepted cell line model of EVT (Graham et al. 1993), transiently expressing different ILK-EGFP fusion proteins (see Chapter 3). The cells were less able to migrate when they were transiently transfected with a dominant negative ILK-E359K. The migration ability of the cells was significantly lower than those cells transiently transfected with ILK-WT and EGFP vector used as a control. Unexpectedly, the cells transfected with ILK-WT did not migrate more than the EGFP-control transfected cells. The lack of increase in number of migratory cells when they were transiently transfected with ILK-WT might reflect that the cells have a high amount of endogenous ILK. The high levels of ILK might be above the limiting rates so increasing the amount of ILK might fail to potentiate ILK-dependent processes. These novel results clearly demonstrated that ILK could indeed mediate trophoblast cell motility. Furthermore, they support a role for ILK in cell migration as shown by studies that reported that SCp2 mouse mammary epithelial cells stably transfected with the wild type form of ILK migrated more than cells stably transfected with antisense ILK (Filipenko et al. 2005). The migration of the SCp2 cells was dependent on the presence of Rac and Cdc42, which would be very interesting to study in the future to elucidate the signaling pathways used by ILK to regulate trophoblast migration. In another study, Vouret-Craviari et al. (2004) showed that in bovine aortic endothelial cells, migration was inhibited when ILK expression was silenced with ILK specific siRNA. They also showed that cell-matrix adhesion was increased in cells lacking ILK and as a consequence migration was significantly

decreased (Vouret-Craviari et al. 2004) demonstrating once more the importance of ILK for cell migration. Fukuda et al. (2003) also showed impaired migration when ILK expression was inhibited in HeLa cells with siRNA specific for ILK.

In an attempt to study the mechanisms underlying the regulation of HTR8-SVneo trophoblast migration by ILK, and considering that the organization of the cytoskeleton can be regulated indirectly by ILK (Zhang et al. 2007), HTR8-SVneo cells transiently expressing pEGFP-ILK fusion proteins were immunostained with EGFP and ILK specific antibodies. The results from these experiments (Fig 3.23) showed that exogenous ILK localized to FAs in cells transiently transfected with pEGFP-ILK-WT but when the cells were transfected with pEGFP-ILK-E359K the ILK immunostaining was primarily cytoplasmic and similar to the cells transiently transfected with pEGFP empty vector. This suggested that ILK-E359K protein was unable to localize to FAs. This might partially explain the lack of migration of the cells into the wound when they are transiently transfected with the dominant negative pEGFP-ILK-E359K since it could sequester key proteins involved in migration away from FA sites. To migrate, a cell needs to be polarized, which means that the molecular processes at the front and at the back of moving cells are different (Ridley et al. 2003; Parsons et al. 2010). It might be that the inability of ILK-E359K to bind to α-parvin (Nikolopoulos and Turner 2002; Stanchi et al. 2009) alters the ability of the cells to form elongated actin bundles at the leading edge of the cell inhibiting the polarization of the cell and in this way impairing cell migration. Although not much is known about the role of α-parvin in actin elongation there are studies that showed that when ILK is unable to bind to parvin, it cannot be recruited to focal adhesions,

which are the specializations that form before the actin bundles are elongated (Zhang et al 2004; Dubash et al 2009). These results are in accordance with Vouret-Craviari et al. (2004), Grashoff et al. (2004) and Sakai et al. (2003) who also found that the absence of normal ILK altered the migration and assembly of fibrillar adhesions in endothelial cells, keratinocytes and mouse embryonic fibroblasts, respectively.

To further study if the cells were able to form fibrillar adhesions when they were expressing the different EGFP-ILK fusion proteins, HTR8-SVneo cells were plated on fibronectin under low serum conditions to induce the invasive phenotype and rhodamine red tagged-phalloidin was added to visualize actin filament bundles. Anti-EGFP antibodies were used to determine if EGFP-fused ILK was localized to those fibrilar adhesions. The cells transfected with the dominant negative pEGFP-ILK-E359K were not able to form fibrillar adhesions in contrast with the control and the pEGFP-ILK-WT transfected cells. This was evident in the latter cells by the presence of elongated structures stained with phalloidin along the side of the cells (Fig 3.24). In the cells transiently transfected with pEGFP-ILK-E359K, the actin bundles were short and were present equally in the cytoplasm and in the plasma membrane where they appeared as non-continuous short lines similar to what Vouret-Craviari et al. (2004) described in endothelial cells transfected with ILK specific siRNA. In cells transiently transfected with pEGFP-ILK-WT, or the pEGFP control, the actin bundles were long and continuous along the plasma membrane. ILK-WT showed a stronger detection than the ILK-E359K in those actin bundles suggesting that ILK localizes to those adhesions. This implicates ILK as an important member of these adhesion specializations in HTR8-SVneo trophoblast cells. Altogether the

results suggested that trophoblasts need functional ILK to form focal and fibrillar adhesions.

Experiments to study the phosphorylation status of AKT following the transient transfection of HTR8-SVneo cells with the different pEGFP-ILK containing plasmids showed that regardless of expression of ILK-E359K, ILK-WT or EGFP alone, AKT was comparably phosphorylated (Fig 3.22 and 5.4). This could mean that ILK does not regulate trophoblast migration through this signalling pathway. Due to the nature of the transient transfection it could also be possible that the endogenous levels of ILK are sufficient enough to phosphorylate the substrates regardless of the exogenous levels of ILK-E359K. Another possible explanation is that the sensitivity of the immunoblot method was not high enough to see a difference in phosphorylation of AKT or GSK3β in response to the expression of the dominant negative ILK-E359K.

The effect of knocking down ILK with siRNA on the migration of the HTR8-SVneo trophoblast cells was also tested (data not shown); however, several attempts to knockdown ILK with siRNA failed, suggesting that ILK was not effectively targeted by the chosen siRNA or that ILK protein levels in the cell were very stable with a long half-life. Due to lack of funding different sequences were not tested. It would be very interesting to develop a future project using ILK knockdown approaches such as siRNA to identify the signalling pathways controlled by ILK during migration. With this approach the endogenous levels of ILK would be minimal and the interpretation might be less complicated.

Hannigan et al. (1996) demonstrated that overexpression of ILK in epithelial cells results in disrupted cell-cell adhesion. ILK can also downregulate β-catenin expression through activation of the transcriptional repressor Snail, independently of β-catenin/T cell factor regulation (Tan et al. 2001). Furthermore, ILK can induce EMT in a variety of epithelial cell types (reviewed in Hannigan et al. 2005). EMT is a critical event in EVT differentiation because these trophoblast cells must undergo EMT in the cell column to facilitate their change in phenotype to less cohesive, single pleomorphic trophoblast cells that will migrate and invade the decidua and maternal spiral arteries (Vicovac and Aplin 1996). Thus, ILK may mediate trophoblast cell differentiation by initiating downregulation of β-catenin expression in vCT and EVT to facilitate the formation of syncytiotrophoblast and induction of EMT. Interestingly, ongoing research in the MacPhee Laboratory has found that Snail and poly (ADP-ribose) polymerase-1 (PARP-1) are expressed in vCT and EVT of human placenta throughout development (Butler et al., manuscript in preparation). It would be very fascinating to study what happens with the expression of these proteins, β-catenin and Snail, if ILK was depleted in trophoblasts with specific targeted siRNAs and migration was inhibited. Since in cancer derived cell lines the overexpression of ILK induced down regulation of E-cadherin by upregulating Snail through PARP-1 (McPhee et al. 2008), it is possible that ILK might be regulating the expression of those proteins to control migration in trophoblast cells.

6.3 ILK Regulates the Invasion of Matrigel in a Trophoblast Derived Cell Line

Differentiation of trophoblasts into invasive cells is crucial for the proper development of the normal placenta. Trophoblasts must acquire the ability to migrate, reach and modify the maternal arteries to transform them into low resistance vessels so enough blood supplies the growing embryo with nutrients and O_2 (Benirschke and Kaufmann 2000). The importance of this process becomes evident when the invasion is inadequate and mothers develop preeclampsia, a syndrome that puts at risk the health of the mother and increases the probabilities of a preterm birth (Goldenberg et al 2008). It is also thought that problems in placentation could be the origins of many future complications for the fetus like cardiac and kidney disease and diabetes mellitus (Barker 1990; Barker 2004). Little is known about what fails during preeclampsia that renders a trophoblast cell unable to invade deeply into the maternal uterus. The finding of which proteins are essential in the process of transforming from one type of cell to another, for example from proliferative epithelial-like trophoblasts to invasive mesenchymal–type trophoblasts, will perhaps provide answers to unsolved problems such as the cause of preeclampsia or the etiology of many miscarriages. It could also lead to the discovery of novel markers for early detection of preeclampsia.

It was hypothesized that ILK regulates invasion in trophoblasts. The results from the invasion assays (Fig 4.8) demonstrated clearly that normal function of ILK was needed in a human derived trophoblast cell line to invade the Matrigel. The

importance of ILK for trophoblast invasion was highlighted by the reduction of invasion when HTR8-SVneo cells were transiently transfected with dominant negative pEGFP-ILK-E359K. This mutant has a point mutation in a conserved glutamic acid residue located in the kinase domain, E359K (Nikolopoulos and Turner 2002) and is unable to interact with paxillin and α-parvin. In contrast to ILK-WT, which co-precipitated both α-parvin and paxillin, no binding of these proteins to the ILK-E359K mutant was observed (Nikolopoulos and Turner 2001). Furthermore, immunofluorescence staining of ILK-E359K expressing cells showed that this mutant cannot target to FAs but remains in the cytosol (Nikolopoulos and Turner 2002). The interaction between ILK and α-parvin is essential for ILK-PINCH and parvin (tIPP) complex formation, recruitment to FAs and stabilization of integrin-actin connection as shown in studies where ILK or PINCH expression were inhibited and FAs did not form (Stanchi et al. 2009). There is evidence that the recruitment of the tIPP complex to integrins is subject to tight regulation that occurs at multiple levels. A first step involves the regulation of tIPP complex formation. A second regulatory mechanism relies on proteasome-mediated degradation of the tIPP components (Fukuda et al. 2003). In this latter study they showed that when ILK or PINCH expression was inhibited the rest of the complex members were degraded by the proteasome system. When ILK is mutated to ILK-E359K, it does not bind to α-parvin (Stanchi et al. 2009), but is still able to bind to PINCH, as the binding site is through the ANK domains in the N-terminal region of the protein (Tu et al. 1999; Chiswell et al. 2008; Maydan et al. 2010). This ILK-E359K might compete with the endogenous ILK, leaving many 'incomplete" complexes in the cytoplasm that are unable to locate to

FAs (Stanchi et al. 2009). Therefore, the mutation might alter the formation of an important signalling node that ultimately drives invasion in the trophoblast cells. Interestingly, the introduction of ILK-S343D into HTR8-SVneo cells significantly increased the number of cells invading the Matrigel (Fig 4.8) while the introduction of ILK-WT resulted in similar invasion to those cells transiently transfected with the pEGFP control vector. These results suggest that ILK kinase activity may be an important factor regulating the invasion of this cell line. Although ILK function has not been explored in trophoblast cells before, these results are in accordance with studies done with cancer-derived cell lines. Inhibition of ILK activity using QLT0267, an ATP-competitive small-molecule inhibitor (Maydan et al. 2010) suppressed the invasive properties of ErbB2-expressing mammary epithelial cells *in vitro* (Pontier et al. 2010). Furthermore, overexpression of ILK in intestinal and mammary epithelial cells resulted in a highly invasive phenotype, which was inhibited by QLT0267 and was associated with increased levels of expression of the matrix metalloproteinase MMP-9 (Troussard et al. 2000). The *in vitro* studies in this thesis confirmed that ILK can regulate trophoblast invasion in the human EVT-derived HTR8-SVneo cell line.

6.4 ILK Expression in Blood Vessels of Chorionic Villi

During the first trimester of placental development, ILK protein was highly detectable in villous stromal cells and was also detectable in developing blood vessels of first trimester and term chorionic villi, where ILK colocalized with vWF, a marker

for endothelial cells (Fig 3.8). It has been shown previously that in both tumors (Tan et al. 2004) and in endothelial cells (Kaneko et al 2004) ILK regulates endothelial morphogenesis induced by VEGF. VEGF is a potent vasculogenic factor. VEGF and its receptor sFlt1, have been detected in villous stromal cells, particularly in the decidual macrophages and in the vCT (Clark et al. 1996; Demir et al 2004). Thus, it is possible that the VEGF response in fetal endothelial cells is mediated through ILK signalling pathways. The transcription factor HIF1α is also detectable at the mRNA and protein levels in villous mesenchyme and trophoblast layers during the early first trimester (Caniggia et al, 2000b; Rajakumar and Conrad 2000) coincident with a low O_2 environment at this time, and is also reportedly detectable in second and third trimester placentas (Rajakumar and Conrad 2000). Expression of VEGF is mediated by HIF1α independently of hypoxia, through a mechanism involving PI3K, ILK, and AKT (Jiang et al. 2001; Fukuda et al. 2002; Tan et al. 2004), and knockout of *Hif1α, Epas1* or *Arnt* in mice resulted in defective placental vascularization in addition to aberrant trophoblast cell fates (Cowden Dahl et al 2005). Gene microarray analysis has shown that ILK expression can be highly upregulated by hypoxic conditions (Scandurro et al. 2001) adding another layer of complexity to the regulation of ILK expression. Finally, endothelial cell-targeted knockout of ILK in mice results in embryonic lethality as a result of defective placental vascularization (Friedrich et al. 2004). Therefore, these reports and our experimental data together strongly suggest that ILK could be involved in placental vascularization during early human gestation.

6.5 ILK is Expressed in the Mouse Placenta

The results from immunofluorescence experiments in the human placenta and migration and invasion assays with HTR8-SVneo cells (Chapters 3 and 4) suggest that ILK plays a major role in migration and invasion of human EVT. To further explore the role of ILK in invasion, and considering the obvious limitations for genetic manipulation that the human placenta presents, the expression of ILK was studied in mouse placenta. The main objective was to examine the expression of ILK in the mouse placenta during development using immunohistochemistry. This information would then become a foundation of knowledge for future exploration of a conditional knockout of ILK in the trophoblast cells that become invasive during mouse placental formation and are likely analogous to populations of invasive trophoblasts in humans. The mouse placenta is a good model because trophoblast cells that form the placenta in the human and the mouse are highly homologous (Georgiades et al. 2002) and the genome of the mouse is well characterized making it possible to manipulate the genetic information. For example, *Mash 2* is expressed in the spongiotrophoblasts and its human orthologue HASH 2 is detected in cytotrophoblasts cell columns (Alders et al. 1997; Janatpour et al. 1999; Hemberger and Cross 2001). In both organisms this gene is also downregulated during trophoblast differentiation into invasive trophoblasts, i.e. the GlyC in mouse placenta and interstitial EVT in humans (Janatpour et al. 1999). *Gcm1* is also expressed in both the mouse placental labyrinth and the human CT in floating villi and has been

implicated in the formation of the syncytiotrophoblast (Cross et al. 2003b; Baczyk et al. 2004).

Studies in early mouse embryo development (Sakai et al. 2003) showed expression of ILK in both the trophectoderm and inner cell mass but until now there was no information available about the expression of ILK in the mouse placenta. Interestingly, this study showed that ILK has a particular temporal and spatial pattern of expression in the placenta during gestation and was expressed in three regions of the mouse placenta (Fig 4.3, 4.4), the labyrinth, the junctional zone and in the TGCs.

The expression of ILK in early stages in the ectoplacental cone (Fig 4.1) suggested that ILK might be present in the cell precursors of the spongiotrophoblasts. By E9.5 it was evident that ILK is highly expressed in the TGCs and to a much lesser extent in the early spongiotrophoblast (Fig 4.2). These differences in level of detection could reflect the different characteristics of the cells: spongiotrophoblast have no invasive characteristics and are supposed to be precursors of other cell types while TGC are fully differentiated cells able to invade the maternal uterus (Georgiades et al. 2002; Simmons and Cross 2005; Simmons et al. 2007).

From E10.5 to E17.5, ILK was expressed in the labyrinth, spongiotrophoblast layer, GlyC, and TGC. In the labyrinth ILK was found in the cytoplasm of cells adjacent to endothelial cells immunostained for laminin (Fig 4.5) which is a marker of fetal capillaries (Natale 2006) and co-localized with Cx26 (Fig 4.6), which suggested that ILK was expressed in the syncytiotrophoblast layer that is adjacent to the fetal capillaries of the mouse placenta (Coan et al. 2005). In contrast to what was observed in human placenta where ILK was highly expressed in the vCT (see Chapter 3), ILK

was expressed in the syncytiotrophoblast layer of the mouse placenta. This is an interesting finding since in the mouse the syncytiotrophoblast is the layer in direct contact with fetal tissues, in contrast to what happens in human placenta where the vCT is lining the fetal mesenchyme, separated by a basement membrane. This could suggest that regulation of ILK expression in polarized epithelia depends on the interaction with the basement membrane or that the expression of ILK might be necessary for the attachment and/or integrity of the basement membrane. There are studies in which keratinocyte-specific deletion of ILK in mice led to reduced integrin-mediated adhesion and impaired basement-membrane integrity (Lorenz et al. 2007). There are other studies that showed that developing mice lacking ILK expression died at peri-implantation stage due to impaired epiblast polarization associated with abnormal F-actin accumulation at sites of integrin attachments to the BM zone (Sakai et al 2003). Recently, it was demonstrated that ILK can facilitate syncytialization and differentiation of human BeWo trophoblast cells (Butler et al. 2009), but the specific role of ILK in the syncytiotrophoblast of the mouse placenta is still unknown. This study now allows a consideration for a syncytiotrophoblast-specific knockout of ILK to specifically determine the necessity and possible function of ILK in this region.

In the junctional zone, ILK was highly expressed in some of the nuclei of the spongiotrophoblasts and in the GlyC (Fig 4.6 and 4.7). Recently it has been shown with double *in situ* hybridization (*Tpbpa and Ascl2,* previously named *Mash2*) that there are at least two populations of spongiotrophoblasts: one group exhibiting *Tpbpa* expression while the other does not (Hu and Cross 2011). It could be that the heterogenous expression of ILK is related to the different origin of the

spongiotrophoblasts or it might be reflecting a different function of ILK in certain spongiotrophoblast cells.

The GlyC are analogous to human EVT cells, particularly the distal cells of the column, which are also rich in cytoplasmic glycogen (Georgiades et al. 2002). In the mouse placenta, GlyC are the only zygote-derived trophoblasts that invade the decidua basalis similar to interstitial EVT in human placenta. They both express urokinase-type plasminogen activator (Teesalu et al. 1998), *Ascl2* (Alders et al. 1997; Tanaka et al. 1997) and IGF-II (Coan et al 2006). Now the results in this thesis show that both also highly express ILK indicating that ILK could be performing similar functions in those cells.

ILK was found in the parietal TGCs that were identified by their large nuclear size and their location, in the region between the spongiotrophoblast layer and the decidua (Fig 4.4). The expression of ILK in the TGC was not homogeneous; some TGC showed higher expression of ILK in the cytoplasm and some showed expression of ILK only in the nucleus. These findings might reflect the different origins of TGCs (Simmons et al. 2007) found in the mouse placenta. Some of the TGCs arise directly from the mural trophectoderm cells in the blastocyst in a process called primary TGC differentiation. However, most of the parietal TGCs arise from the polar trophectoderm and these are known as secondary TGCs (Simmons and Cross 2005). Historically, it has been hypothesized that secondary TGCs are derived from progenitor cells within the ectoplacental cone and the spongiotrophoblast layers that are *Ascl2* and *Tpbpa/4311* positive (Simmons and Cross 2005). However, lineage-tracing studies have shown that only some TGC subtypes arise from *Tpbpa*-positive

precursor cells (Simmons et al. 2007) which are located in the outer ectoplacental cone starting at ~E8.5 and later in the spongiotrophoblast. The source of the *Tpbpa*-negative precursors is unknown but could be the extra-embryonic ectoderm/chorion trophoblast cells, inner ectoplacental cone cells, or both (Simmons et al. 2007). Another possible explanation for the heterogeneous staining could be that ILK might play different roles in TGC and is only translocating to the nucleus in certain stages of the cell cycle. The work in our laboratory, carried out by Trina Butler, demonstrated that nuclear localization of ILK increases in BeWo cells as syncytialization occurs (Butler et al. 2009). By transfecting the cells with a dominant negative pEGFP-ILK-E359K, syncytialization was inhibited suggesting that ILK is necessary for this differentiation process in trophoblasts. It is important to note that, in contrast with human "giant cells", mouse parietal TGCs do not originate from fusion of precursors cells but undergo endoreduplication, a process in which the DNA duplicates repeatedly without division of the cytoplasm (Varmuza et al 1988). Those cells undergo endocycles, instead of leaving the cell cycle, generating a polytenic genome in which many sister chromatids are synapsed together. For cells to switch from a mitotic cell cycle to an endocycle the level of several proteins fluctuate in order to keep the cell in the endocycle without division of the cytoplasm (Follette et al 1998; Zybina and Zybina 1996). For example, mSna, a zinc finger transcription factor homologous to Snail in Drosophila, regulates the G2 decision point of whether trophoblast cells go through mitosis or enter the endocycle (Sigrist and Lehner 1997). It has been shown that mSna is present in the TGC precursors but not in the TGC demonstrating that mSna is a repressor of endoreduplication (Nakayama et al. 1998).

Butler et al. (2009) also showed that the expression of ILK was related to the expression of Snail as increased ILK and Snail expression coincided with repressed expression of E-cadherin during syncytialization. Another difference with the classic cell cycle is that during the transition from the mitotic cell to the endocycle, the cyclin D isoform switches from D3 to D1 (Hu and Cross 2010). It has been shown that ILK regulates the phosphorylation and inactivation of GSK3β, which in turns allows for β-catenin translocation to the nucleus and the increased expression of cyclin D1 (Troussard et al. 1999). So the difference in ILK expression in TGC might be reflecting the presence of cells in different stages of the cell cycle where cells that do not express ILK might have already completed the endocycles. This could be considered analogous to what happens in human placenta where ILK is present in the vCT that are precursors of the syncytiotrophoblast but is barely detected in the syncytiotrophoblast layer.

Overall, the immunolocalization and temporal pattern of ILK expression in the developing mouse placenta confirms the feasibility of undertaking a conditional knockout of ILK in the mouse spongiotrophoblast layer to specifically determine the necessity of ILK for spongiotrophoblast to differentiate into glycogen trophoblast and to regulate glycogen trophoblast invasion.

6.6 ILK Expression at Low Oxygen Tension

ILK gene expression showed a transient rise in expression in HTR8-SVneo cells exposed to low O_2 tension (Fig 5.2). In contrast, HTR8-SVneo cells showed no

detectable differences in ILK protein expression either when cultured under hypoxia mimic conditions created with $CoCl_2$ or DFX (Fig 5.3) or at 3 % O_2 (Fig 5.4). With respect to the different responses observed to a low O_2 environment between mRNA and protein expression, it is possible that post-transcriptional mechanisms could be controlling the expression of ILK. MicroRNAs (miRNAs or miR) are endogenous noncoding RNAs of approximately 22 nucleotides that bind to complementary sequences within mRNAs, thereby either inhibiting translation or causing mRNAs to become degraded (Ambros 2004; Bartel et al. 2004; Ventura and Jacks 2009). Recently a study on c-Src overexpressing tumor cells showed that the down-regulation of microRNA miR-542-3p led to upregulation of ILK which controlled the adhesion and invasion of cells *in vitro* (Oneyama et al. 2012). These results suggested that ILK expression can also be regulated by microRNA expression. miRNA are expressed in a tissue–specific manner (Morales-Prieto and Markert 2011). Recently, a study revealed a group of miRNAs that is expressed by placenta and brain (Miura et al. 2010). miR-542-3p was not included in this list but it would be really interesting to study the presence of ILK regulatory miRNA in the placenta. Regarding the discrepancies observed in the response to the O_2 environment between ILK mRNA and protein expression, it could also be possible that ILK protein turnover could be rapid under these conditions masking any detectable increases following low O_2 exposure. As soon as the cells are exposed to low O_2 there was a significant decrease in the level of mRNA prior to the transient increase observed. A recent study on the stability of ILK estimated that the half life of the ILK protein is approximately 8.4 h in the presence of cycloheximide (Aoyagi et al. 2005), so it could be that under low

O_2 conditions there is a positive feedback (as soon as 6 h upon exposure to low O_2) inducing a transient increase in mRNA to maintain the levels of ILK.

With respect to the difference in ILK protein expression observed between the explant experiments and the HTR8-SVneo cell experiments, where ILK seemed to be downregulated in explants at 3 % and not affected in cell cultures, it could be possible that there are signalling pathways which control the expression of ILK that respond to extracellular matrix signals lacking in cell culture but partially represented in the explant system. For example, growth factors secreted by the mesenchymal cells or the syncytiotrophoblast and/or neighbouring cells in the cell column could be present in explants compared to immortalized cell lines. The differing results could also be a reflection of the different origins in cell types i.e explants that are likely from floating villi (Genbacev et al. 1993a; Miller et al. 2005) versus the cell line HTR8-SVneo, which was derived from EVT (Graham et al. 1993).

It was possible that there was a change in ILK activity instead of a change in level of protein expression in response to low O_2. To test this hypothesis, the expression of pAKT or pGSK3β was first studied by immunoblot analysis as a means of inferring ILK activity. This was evaluated in HTR8-SVneo cells exposed to low O_2 and simultaneously expressing a dominant negative ILK-E359K, ILK-WT, constitutively active ILK-S343D or EGFP alone and compared to cells expressing the same exogenous proteins, but exposed to 20 % O_2. As shown in Fig 5.5, pAKT levels increased in HTR8-SVneo cells exposed to low O_2 tensions suggesting ILK activity could be responsible for this effect, but this increase could not be inhibited by the presence of the dominant negative ILK-E359K. Conversely, there was no increase in

pAKT detection when the cells were transiently transfected with the constitutively active pEGFP-ILK-S343D and exposed to a 20 % or 3 % O_2 environment. It may be that the expression of the fusion proteins was not high enough to compete with the endogenous ILK that still exhibited a high level of detection in transfected cells (Fig 5.5). An alternative way to study if ILK kinase activity was responsible for increased pAKT detection under low O_2 conditions would be to reduce ILK expression by siRNA targetting in a trophoblast-derived cell line such as HTR8-SVneo, expose the cells to a low O_2 environment, and determine pAKT and pGSK3β levels by immunoblot analysis.

When the activity of ILK was studied in HTR8-SVneo cells *in vitro* using GSK3β as a substrate there was no significant change in detection of pGSK3β at 3 % O_2 compared to 20 % O_2 relative to the amount of total GSK3β (Fig 5.6), suggesting that O_2 is not playing a major role in the regulation of ILK activity *in vitro* in HTR8-SVneo cells. These results do not agree with Scandurro et al. (2001) who found that the exposure of the hepatoma derived cell line, Hep3B to 1% O_2 significantly increased the kinase activity of ILK after I h of exposure to low O_2 and this increase was even greater in magnitude at 48 h. The difference in results might be due to differences in the origin of the cell lines studied as Hep3B is a malignant type of cancer-derived cell line and the HTR8-SVneo is immortal but not malignant (Graham et al. 1993). Another crucial difference in the experiments was the percentage of O_2 that the cells were exposed to. In the study of Scandurro et al. (2001) the O_2 tension was 1% and the HTR8-SVneo cells were exposed to 3 % O_2 which is more

representative of the physiological levels of O_2 present in the placenta before week 10 of gestation (Jauniaux et al 1999).

6.7 Preeclampsia

Preeclampsia is associated with failure of cytotrophoblasts to invade the spiral arterioles (Zhou et al. 1993). In this study, a small group of preeclamptic and age-matched control placentas were examined. The placentas from early onset preeclampsia showed a reduced detection of ILK in fetal blood vessels and cytotrophoblasts while the placentas from late onset preeclampsia showed a staining similar to the age matched controls. The lower ILK detection in early onset preeclampsia could be a real reduction in ILK expression that require further study by immunoblot analysis and other techniques like qRT-PCR to study *ILK* gene expression in this tissue. The structure of the preeclamptic tissues looked more similar to a term placenta so it could also be that, as an adaptation to the lower blood supply, the cytotrophoblast cytoplasm becomes thinner to improve the gas and nutrient exchange making ILK detection more difficult by immunofluorescence. Even if that is the case, the expression of ILK in the fetal capillaries also seemed reduced which could suggest that the fetal blood vessels are affected in preeclamptic patients. The villous capillary bed is different from that in systemic organs (Kingdom et al. 2000; Kaufman et al. 2004). From 25 weeks post conception until term, patterns of villous vascular growth switch from prevailing branching angiogenesis to prevalence of non-branching angiogenesis (Kingdom et al. 2000; Kaufman et al. 2004). As a

consequence, the villous capillaries are longer but much less branched, while villous arteries and arterioles are rather short and lack innervation. Thus the villous capillaries contribute more significantly to feto-placental impedance than capillaries do in systemic organs (Kingdom et al. 2000; Kaufman et al. 2004). These characteristics suggest that an alteration in the morphology of fetal capillaries could indirectly affect the maternal blood pressure. The switch in the type of angiogenesis correlates with a decrease in local VEGF and an increase in PlGF (Kingdom and Kaufmann 1999). Studies comparing normal and preeclamptic tissue have shown that levels of PlGF and VEGF are significantly lower than in normal patients. Interestingly, it has been described before that ILK might be regulating VEGF expression in response to O_2 levels (Tan et al. 2004). Since that original report in cancer cells many laboratories have shown that VEGF expression is reduced if ILK is silenced (Edwards et al. 2008; Wang et al. 2011; Wani et al. 2011). Although these studies have been conducted in cancer cells they still suggest that there might be a link between preeclampsia and a lower expression of ILK. What is not possible to know is if the lower detection of ILK in the preeclamptic tissues is a consequence of an unknown alteration that could be impacting ILK levels or if this reduced detection of ILK expression is also playing a major role in the reduction of trophoblast invasion present in preeclamptic women. It would be very interesting to study a larger number of samples of placentas from additional patients and try to detect ILK in plasma and see if the levels of ILK could predict the occurrence of preeclampsia. ILK has already been detected in plasma of patients with invasive ovarian cancer with levels

significantly higher than in normal patients (Ahmed et al. 2004), so a decrease in normal ILK levels would be expected in preeclamptic patients.

6.8 Conclusions and Future Directions

This study showed for the first time that ILK is present in human placenta in the cells that differentiate into syncytiotrophoblast and in cells that differentiate into EVT suggesting an important role in their differentiation. The results from the migration and the invasion assays suggest that ILK directly or indirectly regulates these fundamental processes thought to be responsible for the remodeling of the maternal arteries and the interaction with cells in the decidua. This contributes to the general understanding of normal placental development, a process regulated by a vast number of factors but still poorly understood. Unfortunately, it was not possible to identify the exact pathways that were directly regulated by ILK. The results are still significant as they clearly showed that ILK is important.

To further study the signalling pathways regulated by ILK in trophoblast-derived cell lines, an siRNA approach might prove more useful as, with the right siRNA sequences, very low or no detectable levels of endogenous ILK should be present and therefore the results should be more conclusive than the ones shown here with transient transfection of dominant negative, constitutively active or wild type forms of ILK. The signalling pathway studies should be expanded because it is probable that the regulation of invasion in trophoblast cells is via the regulation of actin cytoskeleton organization or the activation of c-SRC that leads to the activation

of the Ras/MEK pathways instead of the regulation of AKT or GSK3β that regulate survival and proliferation. An array or proteomic approach should render a comprehensive set of data that could be very useful to identify new signalling partners of ILK in the trophoblast.

The fact that ILK was present in the cells that invaded the decidua in mouse placenta, the GlyC, suggested that it was feasible to continue the studies of the role of ILK during gestation with a conditional mouse knockout in the placenta. Thus, a key future step should be to develop mice exhibiting ILK knockdown specifically in the spongiotrophoblast layer that gives origin to the invasive cells in the mouse placenta. The MacPhee laboratory obtained "floxed" ILK mice from the laboratory of Dr. R. Fassler (Max Planck Institute, Martinsreid, Germany) (Sakai et al 2003) and they were crossed with mice that carry the Cre recombinase gene under the control of a spongiotrophoblast specific promoter for the *Tpbpa* gene (Calzonetti et al 1995), obtained from the laboratory of Dr J. Cross (University of Calgary). In this case *Tpbpa* was used because it is expressed in the spongiotrophoblast layer of the mouse placenta and these are the cells that give origin to the GlyC cells (Simmons and Cross 2005). For the establishment of a C57BL/6J mouse line expressing cre recombinase under the control of the mouse *Tpbpa* promoter (*Tpbpa*-Cre+), CD-1 tpbpa-Cre+ mice, provided by Dr. J Cross (University of Calgary, Canada) were crossed with C57BL/6J because this was the strain that carried the floxed *ILK* gene. Eight backcross generations resulted in at least a 99% donor inbred genome. The conditional knockout of ILK in the spongiotrophoblast layer can now proceed and should show if ILK is necessary for the differentiation of spongiotrophoblasts into

GlyC. A TGC specific knockout of ILK could also be conducted due to high levels of ILK in these cells which have an invasive phenotype. These future experiments could have a huge impact on our knowledge of placental development and provide new clues on proteins that could, in the near future, become targets of novel therapies.

ILK detection was reduced in explants exposed to 3 % O_2 and in the fetal capillaries of preeclamptic placentas in contrast with the cell culture system where ILK protein was not affected by a low O_2 environment. This might suggest that, *in vivo* and over time, a down regulation of ILK might render the cells less migratory and invasive leading to failure in placental development. This suggests that the mouse model might prove to be a better model than the trophoblast derived cells for the study of the regulation of these type of proteins that are regulated by the extracellular matrix, growth factors and O_2 in a cooperative manner.

Ideally a higher number of placentas from preeclamptic patients should be compared with age-matched controls to confirm the results obtained in this study. Lastly, another interesting project would be to compare the plasma levels of ILK in normal pregnant women, gestational hypertensive pregnant women and non-pregnant women to test if ILK could be a good marker for the prediction of preeclampsia.

In summary, this is the first study to establish that during early placental development and at term, ILK protein is highly expressed in vCT that will enter the fusion pathway and also in the EVT that has entered the invasive pathway of trophoblast differentiation, and that ILK regulates trophoblast migration and invasion. The results also suggested that the migration might be regulated by the adaptor function of ILK as a dominant negative form of ILK was able to inhibit migration but

was unable to reduce the activation of AKT or GSK3β, two well-known substrates of ILK. During this project, it was also shown for the first time that ILK is expressed in the mouse placenta indicating that it is feasible to conditionally knockout ILK in the different layers of the placenta to test if ILK is necessary for trophoblast differentiation *in vivo*.

These research findings now serve as a foundation for future studies to elucidate the role of ILK in placental vasculogenesis, syncytiotrophoblast differentiation, and the differentiation of invasive EVT. Hopefully it will also contribute to inspire others to continue the quest for markers and potential treatments for preeclampsia, a syndrome that still has no cure other than a preterm delivery.

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