University of Alberta

Mechanisms of Human Placental Growth

By

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DEDICATION:

This thesis and the time and work it represents are dedicated to my husband Kris.

ABSTRACT

The placenta is an essential transitory fetal organ responsible for the key processes of nutrient, oxygen, and waste transfer between the mother and the fetus throughout gestation. Placental size is, importantly, known to correlate to fetal weight, and the malfunction and malformation of the placenta is associated with the common pregnancy complication intrauterine growth restriction (IUGR). IUGR affects ~10% of all pregnancies is defined as a fetus that fails to achieve its genetic growth potential. No curative therapies are currently available for IUGR and a potential target for the development of treatments would be to increase placental growth. Thus, this thesis focused on elucidating mechanisms of two key processes for placental growth: 1) differentiation of the syncytiotrophoblastic epithelium, and 2) extension of the placental blood vessels, or angiogenesis.

Trophoblast differentiation is an essential process in placental growth for the syncytiotrophoblast is a single giant, multinucleate cell, covering the entire surface of the placenta and it is maintained and expands only through differentiation. Previous publications have presented evidence that the initiation of the apoptotic cascade and the externalization of the membrane phospholipid, phosphatidylserine, are required for trophoblast differentiation. However, many of these studies are controversial and were conducted in cell lines. The studies presented in this thesis demonstrate with

primary cells that both apoptosis and the externalization of phosphatidylserine have no role in trophoblast differentiation.

The remaining studies present the identification of a novel population of fibroblastic cells within the human placenta, fibrocyte-like cells, and the ability of these cells to induce placental angiogenesis *in vitro*. It is also demonstrated that fibrocyte-like cells from IUGR placentas have a reduced ability to stimulate *in vitro* angiogenesis and thus may contribute to the malformation of the placenta in the established condition.

In conclusion, the results presented in this thesis are an important contribution to the understanding of the mechanisms of trophoblast differentiation and placental angiogenesis therefore significantly contributing to our understanding of placental growth.

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LIST OF ABBREVIATIONS:

The following abbreviations, definitions, and units have been used throughout this thesis.

Abbreviation	Meaning
DAPI	4',6-diamidino-3-phenylindole, dichloride
8-Br-cAMP	8-bromoadenosine 3'5'-cyclic monophosphate
ARED	absent or reversed end diastolic umbilical artery flow
ATP	adenosine triphosphate
α-SMA	alpha-smooth muscle actin
ATCC	American Type Culture Collection
NH_4Cl	ammonium chloride
ANOVA	analysis of variance
Ang-2	angiopoietin-2
AIF	apoptosis-inducing factor
AGA	appropriate for gestational age
ABCB1	ATP-binding cassette sub-family member B1
BCL9L	B-cell lymphoma 9-like
b-FGF	basic fibroblast growth factor
BSA	bovine serum albumin
BrdU	bromodeoxyuridine
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
CD105	cluster of differentiation 105; endoglin
CD115	cluster of differentiation 115; colony stimulating factor 1 receptor
CD14	cluster of differentiation 14
CD146	cluster of differentiation 146; melanoma cell adhesion molecule
CD31	cluster of differentiation 31; PECAM-1
CD34	cluster of differentiation 34
CD45	cluster of differentiation 45; protein tyrosine phosphatase receptor type C
CD68	cluster of differentiation 68
CD68	cluster of differentiation 68
CD9	cluster of differentiation 9
CD90	cluster of differentiation 90; thymocyte differentiation antigen 1

cAMP	cyclic adenosine monophosphate
cyto-18	cytokeratin-18 neo-epitope
CT	cytotrophoblast(s)
DNAse	deoxyribonuclease
DNA	deoxyribonucleic acid
DMSO	dimethylsulfoxide
EG-VEGF	endocrine gland vascular endothelial growth factor
ELISA	enzyme linked immunosorbent assay
EGF	epidermal growth factor
EDTA	ethylenediaminetetraacetic acid
EVT	extravillous trophoblast(s)
FCS	fetal calf serum
FcCM	fibrocyte-like cell conditioned medium
FACS	fluorescence-activated cell sorting
GCM1	glial cell missing-1
GLUT1	glucose transporter-1
GM-CSF	granulocyte-macrophage colony stimulating factor
GFR-Matrigel	growth factor reduced Matrigel
GEF	guanine-nucleotide exchange factor
HBSS	Hank's balanced salt solution
HGF	hepatocyte growth factor
hCG	human chorionic gonadotropin
hPL	human placental lactogen
HUVEC	human umbilical vein endothelial cells
HIF	hypoxia inducible factor
Ig	immunoglobulin
IGF	insulin-like growth factor
IL-8	interleukin 8
IUGR	intrauterine growth restriction
IMDM	Isocoves Modified Dulbecco's Medium
IMDM-FCS	Isocoves Modified Dulbecco's Medium + 10% fetal calf serum and antibiotics
LGR	Leu-rich-repeat-containing G protein coupled receptors
LEF	lymphoid enhancer factor
MFSD2	major facilitator superfamily domain containing 2
MHC	major histocompatibility complex
MCP-1	monocyte chemoattractant protein-1

NGS	normal goat serum
pfa	paraformaldehyde
PLP	periodate-lysine-paraformaldehyde
PED	persistent end diastolic umbilical artery flow
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween-20
PS	phosphatidylserine
PEDF	pigment epithelial derived factor (Serpin F1)
PEC	placental endothelial cell(s)
PlGF	placental growth factor
PDGF	platelet derived growth factor
PCR	polymerase chain reaction
K+	potassium
KCl	potassium chloride
PE	preeclampsia
PCNA	proliferating cell nuclear antigen
PKA	protein kinase A
siRNA	small interfering ribonucleic acid
$NaHCO_3$	sodium bicarbonate
NaCl	sodium chloride
SGA	small for gestational age
sFlt	soluble Flt-1
SD	standard deviation
ST	syncytiotrophoblast
TCF	T-cell factor
TCF4	T-cell factor 4
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TNF-α	tumor necrosis factor alpha
UEA lectin	*Ulex europaeus* agglutinin lectin
VEGF	vascular endothelial growth factor A
VEGFR-2	vascular endothelial growth factor receptor-2
ZNRF3	zinc and RING finger 3
ZO-1	zona-occludens-1

Mathematical prefixes

K……..	kilo	(10^{3})
d……..	deci	(10^{-1})
c……..	centi	(10^{-2})
m……..	milli	(10^{-3})
μ……..	micro	(10^{-6})
n……..	nano	(10^{-9})
ρ……..	pico	(10^{-12})

Symbols

α……..	alpha
β……..	beta
γ……..	gamma
δ……..	delta
ε……..	epsilon
θ……..	theta

Time units

Mo……..	month
d……..	day
h……..	hour
min……..	minute
s……..	second

Chapter 1: General Introduction

The placenta is, quite simply, the most important organ that you no longer have. Though discarded after birth, often with minimal examination, a properly functioning and well-formed placenta is essential for normal fetal development. The placenta's importance can be highlighted by the fact that trophoblasts, cells unique to the placenta (and a cell type with an entire section of this thesis devoted to it), are the first committed cell lineage found in the developing embryo just three days after fertilization (1). The heart is often stated to be the first organ to develop in the fetus, but committed trophoblast differentiation occurs days before the appearance of cardiac progenitor cells showing that, for the fetus at least, the placenta really is more important than the heart (2). The placenta has many essential roles for the developing fetus: it provides nourishment and oxygen, disposes of waste, maintains water balance, and acts as both an endocrine gland and immunologic barrier, protecting the fetus from the dangers of the maternal immune response. This single organ entirely or partially carries out the functions assigned to the heart, lung, liver, kidney, gut, bone marrow, and endocrine glands of an adult (1).

1.1 Gross Human Placental Structure

The human placenta is discoidal shaped with an average circumference of ~22cm (3) and an average weight of ~650g at term (4). The umbilical cord emanates, most commonly, at the center of the disc from the chorionic plate, or fetal surface of the placenta (1). Besides the attachment of the umbilical cord the other dominant feature of the chorionic plate is the chorionic arteries and veins. The chorionic plate arteries and veins branch from the umbilical cord and run along the plate surface before diving into the villous tissue with arteries crossing over veins (3). From the chorionic plate the human placenta is arranged in a villous form of tree-like projections. It has a highly branched surface with progressively smaller caliber villi emanating from large caliber villi. The villous trees are entirely bathed in the maternal blood of the intervillous space, and the placenta is anchored to the maternal decidua through trophoblastic cell columns at the tips of a specific villous type, the anchoring villi. This multiple branching results in a massive surface area by term. The surface of the villous placenta facing the maternal blood is entirely covered by a single cell, the multinucleate syncytiotrophoblast (ST). The ST first appears at the pre-lacunar stage 7-8 days post coitus after implantation of the blastocyst and is derived from the underlying mono-nucleated cytotrophoblasts (CT).

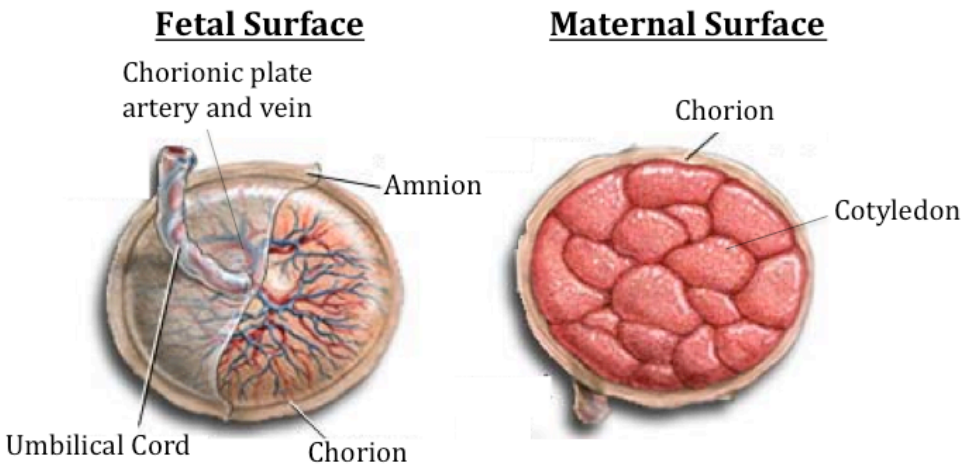

Figure 1-1: Fetal and maternal surfaces of the human placenta
Modified from http://health.allrefer.com/pictures-images/anatomy-of-a-normal-placenta.html

1.2 Villous types and differentiation

1.2.1 Villous types

Placental villi in the first trimester can be categorized into three types of villi: 1° villi contain only CT covered by ST; 2° villi contain mesenchyme which has invaded from the chorionic plate in addition to the trophoblasts; and 3° which also contain capillaries (1,3). By the end of the first trimester all villi can be classified as 3°, with only transitory formation of 1° and 2° villi as the placenta expands through the formation of villous sprouts on the surface of existing villi.

Throughout the rest of gestation tertiary villi undergo differentiation processes to form five different types of villi, with each villous type varying in structure and function.

1.2.1.1 Stem Villi

Stem villi are the largest caliber of the villous types (80-3000μm in diameter)(1). This type of villi is identifiable due to a fibrous stroma and the presence of large centrally located arteries and veins that have established adventia in addition to sparse arterioles and venules (1). Stem villi are found throughout the various generations of villi (from chorionic to basal plate), but have the distinction of being the conduit for the chorionic arteries and veins, thus stem villi always form the primary generation of villous branching (1). Due to the relative absence of small capillaries it is predicted that stem villi have a minor role in maternal-fetal transfer and instead serve primarily as mechanical stabilization (1). Stem villi are also important in the control of placental blood flow for they contain the only muscularized vasculature in the placenta in the form of a continuous myofibroblastic sheath (3).

1.2.1.2 Immature Intermediate Villi

Moving away from the chorionic plate towards the basal surface the next type of villi encountered is the immature intermediate villi. These villi are large (100-400 μm in diameter) and bulbous and are predominantly found in immature placentas. (3). Immature intermediate villi are the progenitors of stem villi therefore they contain non-muscularized arterioles and venules. They also have a highly characteristic stroma that contains long thin mesenchymal stromal cells with long processes arranged into matrix free channels (3,5). These stromal channels are a well-organized network running in parallel to the longitudinal axis of the villi (1,3,5,6).

1.2.1.3 Mature Intermediate Villi

In more mature placentas a third type of villi, the mature intermediate villi, is found in a comparable position to the immature intermediate villi (1). This villous type is long and slender (80- 120 μm in diameter) and contains loose stroma (3,5). In contrast to immature intermediate villi this villous type contains few stromal channels that are short and narrow (5). The vessels in mature intermediate villi are small and located towards the margins indicative of a role in materno-fetal exchange.

1.2.1.4 Terminal Villi

The last villous type is the terminal villi. These are the smallest of the villi, up to 100 μm in length and only ~80 μm in diameter (3). As their name suggests they are the final ramification of the villous tree, branching from mature intermediate villi and infrequently from immature intermediate and stem villi (5). They are the primary site of materno-fetal exchange and as such the stroma is dominated by blood vessels (>50% of the stromal cross sectional area) (3). Terminal villi contain highly characteristic sinusoidally dilated vessels that are found in extremely close contact with the overlying ST forming the aptly named vasculosyncytial membrane (1,3,5,7).

1.3 Cellular components of the villous placenta

The cellular components of the villous placenta can be roughly subdivided into four classifications: 1) Trophoblastic; 2) Endothelium; 3) Fibroblastic stroma; and 4) Placental macrophages.

1.3.1 Trophoblasts

As already introduced briefly above, the trophoblast is a cell type unique to the placenta. These are the first committed cell lineage in an embryo and the only placental cells in direct contact with both maternal blood and tissue. There are two different lineages of trophoblasts in the placental: villous and extravillous. Villous trophoblasts reside exclusively in the villous tissue and are epithelial cells that are separated from the villous stroma by a basement membrane (1,3). Extravillous trophoblasts (EVT) are predominantly found outside the villous placenta with the majority of EVT found within the maternal decidua (1). EVT are primarily an invasive cell type which are responsible for the invasion and remodeling of maternal spiral arteries. The process of remodeling the spiral arteries from low volume high resistance vessels to high volume low resistance vessels is an important process during pregnancy ensuring adequate delivery of oxygen and nutrients to the developing fetus. Deficient remodeling of the maternal spiral arteries is hypothesized to lead to the establishment of the common pregnancy complications preeclampsia and intrauterine growth restriction (IUGR).

1.3.1.1 Syncytiotrophoblast (ST)

The ST is a giant multinucleate cell that exclusively forms the surface of the villous placenta. Importantly, there is only a single ST per placenta with no intracellular borders identifiable. During the first trimester there is a seemingly even distribution of nuclei and organelles within the syncytium, but by term there is considerable variability in the concentration of organelles throughout this massive cell (3). It is completely unknown how this variable concentration of organelles develops and is maintained as well as whether specific organelle compositions are found in comparable locations throughout the placenta (for example in villi that lie close to the periphery). Being an epithelial cell the ST possesses differentiated basal and apical surfaces. The apical surface is a characteristic brush border membrane and is highly microvilliated. This microvilliation functionally increases the surface area of the cell, an estimated $12m^2$, by a factor of 7.67 (1). This arrangement is therefore ideal for maternal-fetal exchange.

Despite the lack of intracellular borders the ST clearly displays specialized regions when examined by transmission electron microscopy. In fact, it has been subdivided into three zones (8): the absorptive, secretory, and basal zones. The absorptive zone has been designated as the apical surface of the ST, an area containing small and large coated vesicles. Below this in the central third of the ST has been designated secretory zone and contains large amounts of rough endoplasmic reticulum. The basal third of the ST was

simply labeled as the basal zone and has been described as having organelles resembling those found in the underlying CT. Additionally, several specialized regions have been designated within the syncytium based on their thickness, the abundance and identity of the intracellular organelles, and the abundance of nuclei present by TEM. Two such regions are designated based on the type of endoplasmic reticulum present. Regions containing abundant rough endoplasmic reticulum are the most common specialized region within the ST (1). These areas are of varying thickness, may or may not contain nuclei, and also contain other intracellular organelles such as Golgi apparatus, abundant mitochondria, and many lysosomes. Immunohistochemical studies have shown that rough endoplasmic reticulum regions are heavily involved in the synthesis of hormones such as human chorionic gonadotropin (hCG), human placental lactogen (hPL), oxytocin, erythropoietin, corticotropin-releasing hormone, pregnancy specific glycoproteins, and leptin (1,9-14). The ST is a highly secretory cell that is also responsible for the production of progesterone, estrogen, and placental growth hormone (15). Rough endoplasmic reticulum regions have also been shown, again by immunohistochemistry, to be sites of energy metabolism enzyme production (e.g. lactate dehydrogenase) (1).

Regions containing predominantly smooth endoplasmic reticulum are also found within the ST. These areas are small and found as islands within large

rough endoplasmic reticulum regions. Immunohistochemical evidence suggests that these regions are involved in steroidogenesis (1).

The remaining specialized regions within the ST are designated based more on their implied function and the accumulation of nuclei. Vasculosyncytial membranes, found exclusively in regions of ST covering terminal villi, are also designated as a unique region. These regions are very thin (0.5-1μm) and as such are devoid of nuclei and other intracellular organelles. As previously discussed these areas are clearly sites meant to facilitate materno-fetal exchange for they can be so thin that the basement membranes of both cell types actually become fused (3).

In stark contrast to the vasculosyncytial membrane, syncytial knots are specialized regions containing an abundance of nuclei. These nuclei are at various stages of chromatin condensation, though predominantly display heterochromatin, and because of the predominance of highly condensed DNA are believed to be undergoing apoptosis (16). Syncytial knots are devoid of other intracellular organelles and contain very little cytoplasm. It is somewhat controversial whether these areas do represent a cluster of apoptotic nuclei and if they are in fact pinched off of the ST and released into the maternal blood, as widely stated in the literature, for the highly condensed nuclei in syncytial knots almost never display nuclear blebbing (a hallmark of the apoptosis process) (1,16). It has instead been proposed that

these areas are sites of sequestration of transcriptionally inactive nuclei and that the nuclei found in the maternal circulation are instead from ruptured syncytial sprouts (areas of new ST outgrowth), though much work must be completed in this area to clearly understand the purpose and regulation of syncytial knots (16).

It has not been established whether ST produced and maintained *in vitro* (i.e. in explant culture or through differentiation of isolated CT) are also separated into these distinct zones and regions. Syncytial knots are commonly studied using explant culture of first or third trimester placentas, but whether other important regions, such as rough endoplasmic reticulum regions are present has never been examined (16-19). Since these specialized regions may have some regulation over trophoblast differentiation morphologic studies of *in vitro* produced syncytium would be an interesting direction for trophoblast research to explore and a powerful tool to examine the involvement of different organelles in the differentiation process.

A final key characteristic of the ST is that despite its massive size and an abundance of intracellular organelles it does not undergo cellular replication. This important feature was initially established using the Feulgen staining method to examine mitosis and DNA content in first trimester CT and ST. Using this method mitosis was exclusively observed in the CT and the ST nuclei were always diploid (20). This observation has been replicated

countless times and proliferation markers such as Ki67, proliferating cell nuclear antigen (PCNA), and bromodeoxyuridine (BrdU) incorporation are never observed in the ST (21-23). Thus it is accepted that this behemoth of a cell is maintained and expands only through the incorporation of its stem cells, the CT.

1.3.1.2 Cytotrophoblasts

Villous CT reside directly below the ST basal membrane and in contrast to their colossal neighbor are replicative, mononucleate cells. Ultrastructurally, CT contain few mitochondria and rough endoplasmic reticulum, but abundant polyribosomes. A small proportion of CT display "ST like" characteristics with abundant rough endoplasmic reticulum, polyribosomes, and mitochondria filling their cytoplasm (24). This "ST like" phenotype is thought to represent a population of CT that have begun to differentiate and are preparing to fuse and become incorporated with the ST. CT always reside on top of the trophoblastic basement membrane which separates the trophoblastic epithelial layer from the underlying stroma.

Approximately 50% of CT at any one time have been shown to be replicating through expression of cyclin D1, Ki-67, and ^{3}H-thymidine incorporation (21,25,26). During the first trimester CT cover ~90% of the trophoblastic basement membrane and are cuboidal in shape, but by term CT coverage becomes sparse (~44% coverage) and the cells become thin cells with many long processes (27-29). The reduction in coverage is not due to a reduction in

CT numbers though for the absolute numbers of CT have been shown to rise throughout gestation (1).

1.3.2 Placental Endothelium

Placental endothelial cells comprise a large proportion of the cellular constituents in the villous placenta. Next to the trophoblast, they are the most abundant cell type in the smaller caliber villi and are an obligate component of all villi after the first trimester. Placental endothelial cells (PEC) are of a non-fenestrated phenotype and the placenta contains both micro- and macro-vasculature. Differential expression of established endothelial cell markers has been observed with immunohistochemistry between the macro- and micro-vasculature in the villi namely, von willebrand factor, occludin, claudin-1, and the vasodilator endothelial nitric oxide synthase are more intensely expressed in the macro versus micro-vasculature (30). Evidence of endothelial cell proliferation through immunohistochemical analysis of PCNA has shown that proliferation is limited to the microvasculature within the mature intermediate villi and the terminal villi (30). Adjacent PEC are linked through both tight and adherens junctions (24,31) and the endothelial basement membrane has been shown to contain fibronectin, collagen type IV, laminins, and heparan sulfate proteoglycans by immunohistochemistry (24,32).

1.3.3 Fibroblastic Stroma

The abundance and diversity of fibroblastic stromal cells differ depending on the villous type in which they are found. Cells with classical fibroblastic ultrastructure (high amounts of rough endoplasmic reticulum and short cytoplasmic processes) are found exclusively in the stem villi beyond the 15th week of gestation (5). Ultrastructural analysis has shown that beyond the first trimester myofibroblasts, fibroblastic cells with apparent contractile abilities, are also found exclusively in the stem villi in close proximity to the vasculature (33), whereas the immature and mature intermediate villi as well as terminal villi that have fibroblastic stroma contain reticulum cells (5). This observation has been confirmed with immunohistochemistry using alpha-smooth muscle actin (α-SMA) as a marker for myofibroblasts (34). The reticulum cell has a uniquely identifiable ultrastructure amongst the fibroblastic stroma for they have a large nucleus, few rough endoplasmic reticulum, distended Golgi apparatus, and a small cell body with richly branched cytoplasm (5). They have also been observed to be irregularly shaped with sail-like processes and were shown to line the stromal channels which are characteristic of immature intermediate villi. The final type of fibroblastic stroma identified by transmission electron microscopy studies are mesenchymal cells. These cells are only found in the first trimester in mesenchymal villi. These are small cells (10-20 μm long and 3-5 μm wide) with a small cell body and cytoplasmic processes (5,6).

Fibroblasts are in general and no matter the tissue origin an under-characterized cell type. This is due to the difficulties in identifying specific populations, for no single marker has been characterized that definitively identifies a fibroblast. Thus examination of, what is becoming increasingly clear from studies in organs such as the heart, the diverse population of cells that are all simply called fibroblasts is difficult. In recent years the origin of fibroblasts has been shown to be diverse. For example, cardiac fibroblasts are now known to be derived from resident fibroblast populations, from the endothelium via endothelial-mesenchymal transition, from monocytes, from perivascular cells or pericytes, and from a population of cells called fibrocytes (a circulating population of cells which express both hematopoietic and mesenchymal cellular markers) (35). Due to the lack of specific markers for fibroblasts some common non-specific markers used to identify this cell type are α-SMA, fibroblast activation protein-1, fibroblast surface antigen, CD90, and vimentin (35). In the placenta CD90, α-SMA, vimentin, and CD9 have commonly been used to identify fibroblasts (34,36,37). As already introduced above α-SMA can also be used to identify myofibroblasts, therefore a certain amount of confusion can arise due to the non-specific nature of common markers. When examining evidence collected using these common markers and when taking the ultrastructural data presented above into account it becomes clear that distinct populations of fibroblasts likely exist in the placenta, but no immunohistochemical identification schemes have thus far been developed. Essentially all of our

knowledge about potential functions and specialization within the fibroblastic stroma is derived from immunohistochemical examination of specific growth factors and the expression patterns of receptors and other proteins. Studies specifically aiming to understand potential functions of the stroma are very limited. It is apparent that the fibroblastic stroma is important for mechanical stability of the placenta, that it is the primary source of extracellular matrix production and a site of production for a wide array of growth factors as well as other regulatory proteins controlling essential processes such as angiogenesis, trophoblast differentiation, and maintenance of a stem cell niche (1,34,37-44).

Several studies have also been published with isolated placental fibroblasts examining their growth under hypoxia (45), production of inducers of trophoblast differentiation (37), and collagen and extracellular matrix production (44). Since each set of authors uses their own markers or simply cellular morphology and slightly different methods to isolate placental fibroblasts it is likely that many of these studies are examining slightly different and/or heterogeneous populations of fibroblastic cells. This literature is only becoming increasingly confounded due to the popularity of the placenta as an organ for the derivation of cells with multi-lineage differentiation potential such as mesenchymal stem cells and mesenchymal stromal cells. Again each set of authors has their own specific set of markers that they examine as well as different isolation protocols. Unfortunately

papers of this sort seem to struggle to differentiate between a decidual, placental, umbilical, and amnion/chorion origins of the cells so careful examination of the methods and not a simple read of the abstract is required.

The multiple cell types of the fibroblastic stroma in mature placentas have been predicted to differentiate from mesenchymal cells (the only fibroblastic stromal cell population identified in the first trimester by ultra-structural analysis) (5,24). This is likely an overly simplistic hypothesis though for despite their ultra-structural similarity mesenchymal cells are very likely to be made up of multiple cell types. Mesenchymal stem cells have been shown to reside in a vascular niche directly surrounding the PEC at term (39,42) and have been isolated from first trimester samples (though their localization was not examined in these early samples) (46). Thus differentiation from mesenchymal stem cells is likely to occur, though there is not yet direct experimental evidence for this occurring in the placenta. This same vascular niche that supports mesenchymal stem cells has been shown to contain hematopoietic stem cells in mice and it is likely that human hematopoietic stem cells are found in similar proximity to the vasculature (38). Hematopoietic stem cells are not likely to directly contribute to the development of the fibroblastic stroma but are a source of placental macrophages. There is growing evidence for the trans-differentiation of macrophages to form fibroblastic cells and cultured placental macrophages have been shown to rapidly change to a fibroblastic morphology in culture

(35,47). Therefore this is another potential pathway for the development of certain populations of placental fibroblasts. Pericytes are fibroblastic-like cells that are embedded in the capillary endothelial basement membrane and have been shown to have multi-lineage differentiation potential. These are also a likely source of placental fibroblastic stroma in some villi although they are absent from much of the smaller caliber villi of the placenta (42,48,49). Finally, the fibroblastic stroma may also be derived from fibrocytes. Fibrocytes, or stellate cells that express both hematopoietic lineage markers (CD45, CD34, major histocompatability locus-II) as well as stromal cell markers (production of collagen), are a circulating population of cells that are a known source of fibroblasts in adult tissue (35). Fibrocytes have been identified in the umbilical cord and chorionic plate arteries and are therefore likely circulating through the placenta, and may contribute to the production of placental fibroblasts (50).

Due to the expected importance of the fibroblastic stroma in key aspects of placental biology the origin, function, and exact constituents that make up this important population of cells requires much attention.

1.3.4 Hofbauer Cells

The final major cellular constituent of the villous placenta and the only resident immune cell is the placental macrophage, or Hofbauer cell. These cells are easily identifiable by transmission electron microscopy for they are large rounded pleiomorphic cells with a highly vacuolated and granular

cytoplasm (1,47). The macrophage identity of these cells has been confirmed *in vivo* due to their expression of various monocyte/macrophage lineage markers such as CD68 and CD14 as well as the hematopoietic lineage marker CD45. Hofbauer cells have first been shown to be in the villous stroma during the fourth week of development with their relative numbers per unit area increasing to a maximum by week 18 (47). These cells are therefore hypothesized to play a role in villous stromal formation due to their early emergence and localization at this early time point (47). An interesting detail that remains to be unequivocally established is whether the earliest Hofbauer cells differentiate from hematopoietic stem cells within the placenta or whether they are instead derived from a pool of progenitors within the yolk sac.

As they are macrophages Hofbauer cells are phagocytic and they are predicted to be involved in placental immune defense, tissue remodeling, and water balance maintenance (3,6,47). A role in the formation of *de novo* blood vessels, or vasculogenesis, has also been hypothesized due to the proximity of Hofbauer cells to developing angiogenic cell cords and their high expression of vasculogenic/angiogenic growth factors (51,52). Additionally fetal macrophages in mice have been shown to be actively and directly involved in the anastomosis of new vessels, thus the role Hofbauer cells play in placental angiogenesis is a key area for future research (53).

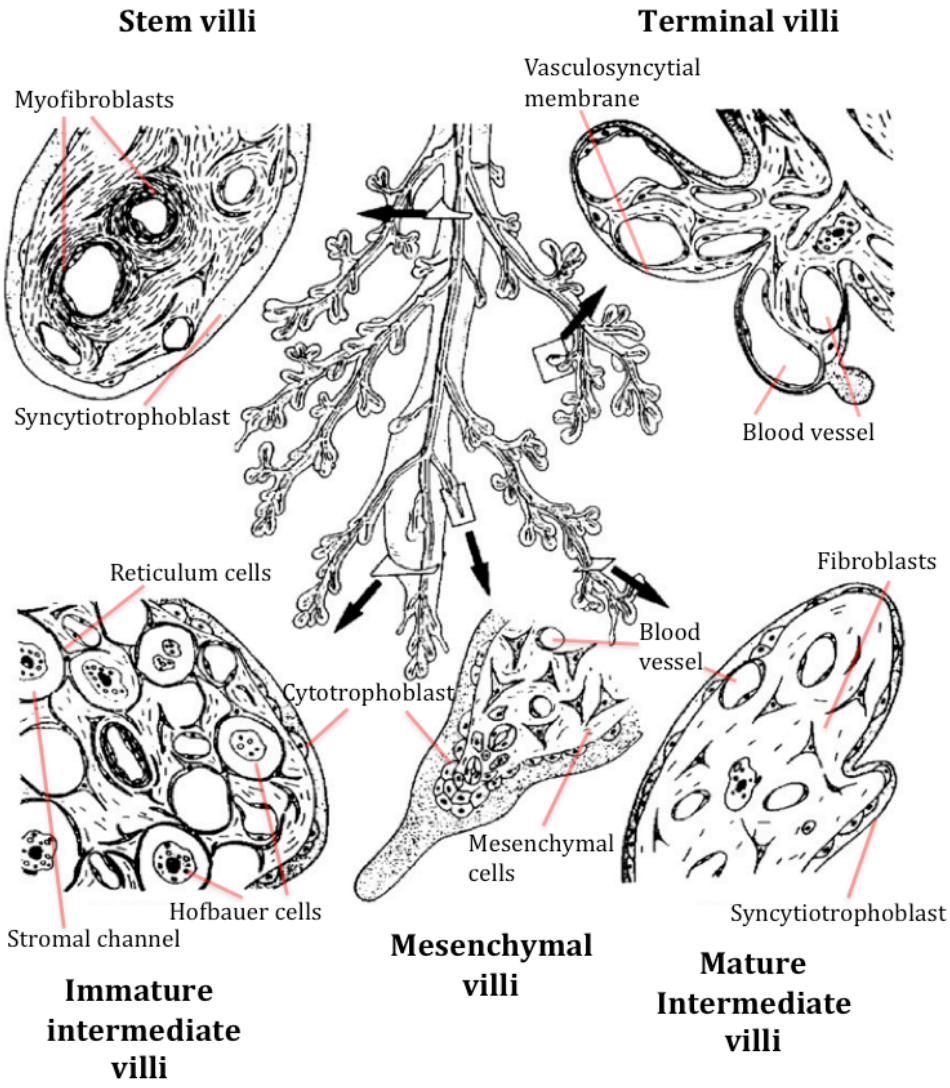

Figure 1-2: Villous types of the human placenta
Modified from Fox H.; Aging of the placenta. Arch. Dis. Child. Fetal. Neonatal Ed. 1997; 77 (3): F171-5.

1.4 Placental Growth

It is generally accepted, based largely on experimental models of placental reduction in sheep, that placental weight and fetal weight are directly related (54). The direct relationship between fetal and placental weight can be clearly evidenced in humans in small for gestational age (SGA) infants and the common pregnancy complication of intrauterine growth restriction (IUGR). SGA infants have been found to have a 24% reduction in placental weight compared to appropriate for gestational age infants (AGA) and that the actual placental weight is lower in SGA infants compared to AGA infants of the same birth weight (54). Heinonen et al. defined SGA in this study as a sex and age-adjusted birth weight below the tenth percentile for gestational age, therefore it is likely that that this group contained infants that had achieved their genetically predetermined growth potential, and were not pathologically small, as well as those that did not achieve their genetic growth potential and could be designated IUGR. Thus providing evidence that both of these conditions have reduced placental size.

Placental growth is primarily accomplished during the first 20 weeks of pregnancy in contrast to fetal growth that is exponential in the final trimester, therefore proper development and growth of the placenta seems to be essential for optimal fetal growth (55). There are two essential processes that are required for placental growth: 1) expansion of the ST

through differentiation and 2) expansion of the placental vascular network through angiogenesis.

1.4.1 Villous Trophoblast Differentiation

As the ST is incapable of proliferation and covers the entire surface of the villous placenta ST expansion is a key checkpoint in the overall process of placental growth. The micro-environmental conditions that stimulate the initiation of trophoblast differentiation largely remain to be discovered and clues as to which cell types control this essential process are only just beginning to be understood. There are two general stages of trophoblast differentiation. Firstly there is fusion, which requires the initiation and up-regulation of signaling and the fusion associated cellular machinery culminating in the physical joining of the CT and ST membranes. Secondly there is the functional differentiation of the CT to become part of the ST. This stage of differentiation likely includes the trafficking of CT intracellular organelles to areas throughout the ST and the functional alterations of these intracellular organelles to perform ST instead of CT activities (i.e. hormone production and ST specific protein production).

1.4.1.1 Models to Examine Trophoblast Differentiation

Currently all of our knowledge regarding the molecular signals that control trophoblast differentiation comes from *in vitro* models. The three most common models in use are: 1) the Bewo choriocarcinoma cell line; 2) Isolated primary CT; and 3) Placental explant models.

1.4.1.2 Bewo Choriocarcinoma Cells

The Bewo choriocarcinoma model is commonly used to examine molecular control of cellular fusion and the functional differentiation of trophoblastic cells (i.e. production of hCG). This model is advantageous for examining cellular fusion for fusion must be induced and almost no spontaneous fusion events occur (56). Bewo fusion is most commonly induced with the adenylate cyclase activator forskolin, which can result in incorporation of up to 80% of the nuclei into multinucleate syncytia, but depending on the conditions can result in fusion in as few as 20% of the cells (56,57). Due to the transformed nature of Bewo the cells are also easily transfectable and thus an excellent model for the manipulation of protein expression (58). The transformed nature of the cells is also the major drawback of this model. Studies have shown over-expression of the tumor suppressor proteins p53, $p21^{WAF1/CIP1}$ and Rb as well as the oncogenes erbB-2 and mdm-2 by immunohistochemistry, western blot and northern blot analysis as well as increased telomerase activity (59). All of these proteins are involved in cell cycle regulation, differentiation and senescence. Additionally, Bilban et al. (2010) recently published a bioinformatic comparison of primary villous CT compared to Bewo and several other cell lines comparing gene expression of 14, 000 genes (60). This study found that the gene expression profile of primary CT was, not surprisingly, distinct compared to the choriocarcinoma cell line. Thus alterations in the molecular pathways that regulate

differentiation in Bewo are likely and often overlooked and are a key limitation to utilization of Bewo as a model.

1.4.1.3 Isolated Primary Cytotrophoblasts

In contrast to Bewo usage of primary trophoblasts for examination of differentiation avoids the usage of transformed cells therefore minimizing the caveat of potentially altered differentiation pathways. Primary CT fuse robustly with and without stimulation and are therefore an excellent model to examine fusion and functional differentiation. Spontaneous fusion occurs in ~ 40% of CT after 72h in culture under most conditions compared to stimulated fusion rates between 20-80% with Bewo (57,61,62). Additionally, higher rates of fusion (up to 70-85%) can be stimulated with forskolin, cell permeant forms of cAMP such as 8-Br-cAMP, granulocyte-macrophage colony stimulating factor (GM-CSF), epidermal growth factor (EGF), adiponectin, and hormones such as hCG, estradiol, and dexamethasone (61,63-68). The primary CT model does not come without drawbacks as well though, namely isolation procedures are very labor intensive and CT proliferate at extremely low rates *in vitro* (<5% of CT stain for Ki67 by flow cytometry after 24h in culture) therefore cells from many different patients must be used leading to headaches when it comes to data analysis due to natural biological variation (69). This low proliferation rate also excludes examination of how the CT leaves the cell cycle in preparation for fusion. Another concern with this model is attaining high cell purity in order to minimize the chance that contaminating cells have an effect on trophoblast differentiation. This is quite

easily controlled by staining for vimentin positive contaminating cells and the purity achieved by most isolation techniques ranges from >90 to >99% pure (62,69,70). Primary trophoblasts are also difficult, though not impossible, to transfect. Development of new transfection reagents and techniques has made molecular manipulation of these primary cells much easier and transfection has now been clearly achieved (71).

1.4.1.4 Villous Explant Models

The final model commonly used to examine trophoblast differentiation is the villous explant model. Villous explant models have the major advantage over isolated cell culture of maintaining cell-cell contacts and morphological arrangement. The original ST layer is usually shed within the first 48 hours of culture therefore most of the ST observed after >72 hours in culture is due to differentiation that has occurred *in vitro* (72). Complete denudation of the syncytium can be achieved with trypsin treatment of the explants upon culturing. The major drawback to the explant model is the quantitation of differentiation. Subtle changes in the rate of differentiation are very difficult to detect and a high degree of variability in the amount of ST that falls off or is denuded requires the proper controls to determine the quality of each experimental run. As with primary CT models a high degree of biological variation between samples can also mask subtle changes.

1.4.2 The Steps of Differentiation

As outlined above there are essentially two major steps to differentiation: fusion and functional differentiation. Within these two steps there are many individual processes that must occur in order for either of these events to be successfully completed.

1.4.2.1 Acquisition of Fusion Competence

Fusion is undoubtedly a many-staged process culminating in the final event of the combining of membranes of the CT and ST. Initially CT and ST must be made to become "fusion competent." Cellular fusion events must be tightly regulated to avoid erroneous fusion between the wrong cell types. Hence, there must be specific cellular pathways that tell individual CT and specific areas of the ST to ramp up the machinery required for cellular fusion. There is morphological evidence for this step of differentiation on the CT side, for specific CT have intracellular organelles that are more reminiscent of the types and proportions found in the ST than their sister CT (1,24), but there is no evidence thus far as to how the ST enters this fusion competent state. The acquisition of fusion competence likely involves changes in the expression of membrane proteins (the machinery of membrane fusion) for both the CT and ST. Most of the work that has been done thus far in the field of trophoblast differentiation examines the various stimuli and molecular pathways involved in the acquisition of fusion competence.

1.4.2.1.1 Wnt/β-catenin signalling

The Wnt/β-catenin signaling pathway is an evolutionarily conserved pathway that regulates embryonic axis formation, stem cell self renewal, organogenesis, and has recently been shown to regulate trophoblast fusion (73,74). There are many different Wnt receptors and ligands as well as multiple signaling pathways but the best characterized is the canonical Wnt/β-catenin signaling pathway (74). With Wnt activation β-catenin is stabilized within the cytoplasm of the cell and enters the nucleus where it interacts with transcription factors from the T-cell factor (TCF)/lymphoid enhancer factor (LEF) families and leads to the transcription and expression of Wnt target genes (73,74). Multiple cofactors are required for β-catenin transcriptional activity and Matsuura et al. (2011) have found that Wnt/β-catenin signaling in association with the B-cell lymphoma 9-like (BCL9L) cofactor is important for trophoblast fusion (73). The authors found that mouse models lacking *bcl9l* expression fail to develop a proper labyrinth layer (the equivalent of the human villous placenta) with a complete absence of cellular fusion in the trophoblastic cells surrounding the fetal capillaries visible by electron microscopy. They additionally show that siRNA mediated knockdown of BCL9L, β-catenin, or T-cell factor 4 (TCF4) leads to a 50-90% decrease in Bewo cellular fusion when fusion is stimulated with forskolin. The authors are able to show that BCL9L/β-catenin/TCF4 influence cellular fusion directly through glial cell missing-1 (GCM1), a key transcription factor in trophoblast fusion. Additionally they hypothesize that BCL9L/β-

catenin/TCF4 may work with the frizzled5 receptor and the R-spondin 3 signaling activator. The involvement of R-spondin 3 seems particularly promising for *R-spondin 3* knockout mice have significantly decreased GCM1 expression. Frizzled5 receptor expression in human tissue has been analyzed by semi-quantitative PCR and it was found to be strongly expressed in isolated first and third trimester CT as well as whole first trimester placenta, but expression in intact third trimester placenta was absent (76). R-spondin 3 is highly expressed in the term human placenta though to date no studies examining which cells express the protein or the expression levels at other stages of gestation are available (77). Studies utilizing human tissue to further examine the role these proteins may play in controlling GCM1 are an exciting future avenue of research.

1.4.2.1.2 Glial cell missing-1

As mentioned above, a key transcription factor involved in the acquisition of fusion competence appears to be glial cell missing-1 (GCM1). This transcription factor is essential for the development of the mouse placenta for GCM1 knockout mice fail to develop ST and are thus embryonic lethal due to failure of development of the placental labyrinth (78). GCM1 silencing in Bewo as well as villous explants lead to a reduction in fusion and an increase in proliferation of CT (79). Importantly knockdown of GCM1 also blocked EVT differentiation in villous explants thus it is likely a regulator of both lineages. Interestingly GCM1 expression could not be detected by western blot in primary cells with the same antibody that detected high levels in

placental explants (80). The fusogenic effects of GCM1 are proposed to be mediated by the HERV-W retroviral protein syncytin-1 (81).

1.4.2.1.3 Syncytin-1

Syncytin-1 is a highly fusogenic membrane glycoprotein which has been shown to interact with the type D mammalian retrovirus receptor (82). Transfection of syncytin-1 into a variety of cell lines resulted in syncytia formation (82) and syncytin-1 has been shown by immunofluorescence to be highly expressed on extended cellular processes at sites of cell-cell contact in both Bewo and primary CT (83). Importantly this localization pattern was not observed in Bewo that had not been stimulated to fuse. siRNA knockdown of syncytin-1 in primary trophoblasts lead to a 50% decrease in fusion and a 30% decrease in fusion in Bewo (83). After an initial up-regulation of the protein when Bewo are stimulated with forskolin syncytin-1 expression has an inverse relationship to the proportion of cells that are fused (83).

1.4.2.1.4 Syncytin-2

A second HERV-W protein syncytin-2 has also been shown to be important in membrane fusion and thus the acquisition of fusion competence. Syncytin-2 is specifically expressed by the CT and its receptor, major facilitator superfamily domain containing 2 (MFSD2), is specifically expressed by the ST (84). siRNA reduction of syncytin-2 in primary trophoblasts lead to a 90% reduction in fusion and a 60% reduction in Bewo (83). In contrast to

syncytin-1 that is focally expressed, syncytin-2 is expressed throughout the cell membrane and expression levels of syncytin-2 correlate more closely with fusion than those of syncytin-1 (83). Thus both of these endogenous retrovirally transcribed proteins are likely to be key players in readying the CT for fusion.

1.4.2.1.5 Gap Junctional Proteins

Gap junctional communication between cells has also been implicated in the acquisition of fusion competence. Specifically, the expression of the gap junction protein connexin 43 has been shown to increase at the contact areas between trophoblasts *in vitro* as fusion increases. This is concomitant with a decrease in the peripheral membrane protein zona-occludens-1 (ZO-1) that has been shown to functionally interact with connexin-43 during differentiation by co-immunoprecipitation (85). siRNA knockdown of ZO-1 lead to a decrease in the expression of connexin-43 and to a decrease in the rate of fusion (85). It is thought that the connexin-43/ZO-1 unit allow for rapid communication between the cells that are going to fuse allowing synchronization of key processes that will allow fusion to occur. Phosphorylated connexin-43 has also been shown to co-immunoprecipitate with the syncytin-1 receptor SLC1A5 and co-localize with SLC1A5 to areas likely undergoing CT fusion in explant models (86).

1.4.2.1.6 Caspase 8

The final protein that has been implicated in this first step of trophoblast differentiation is not a membrane protein at all. Caspase 8 is a cytoplasmic cysteine-aspartic pro-enzyme which is part of the classical apoptotic pathway of cell death. Multiple caspases exist and can be classified as initiator caspases (those involved in the initiation of the apoptotic pathway; caspase-6, 8, 9) or executioner caspases (those involved in the downstream execution of the apoptotic pathway; caspase- 2, 3, 7) (87,88). Caspase 8 is known to induce mitochondrial outer-membrane permeabilization thus directly leading to apoptosis (89). The apoptotic functions of caspase 8, and all caspases require proteolytic cleavage of the pro-enzymes and caspase 8 is sequentially cleaved from a 56kDa pro-form to active 18 and 10 kDa forms (89). Black et al. (2004) presented evidence that activated caspase-8 was required for trophoblast fusion (90). Using anti-sense oligonucleotides and the peptide caspase-8 inhibitors zIETD-fmk and IETD-CHO in placental explants Black et al. showed an accumulation of CT nuclei and an absence of newly produced ST. Thus they concluded that activated caspase-8 is required for trophoblast fusion. This observation in conjunction with the observation that anti-apoptotic proteins Bcl-2 and mcl-1 are up-regulated during differentiation was then used to postulate that apoptosis initiation in the CT is required for trophoblast differentiation (91-94). This hypothesis holds that apoptosis is initiated in the CT, or more specifically that the activation of the apoptotic cascade through caspase-8 activation occurs.

Cellular fusion can then occur and apoptosis is inhibited by the abundance of Bcl-2 and mcl-1 expressed in the ST until the accumulation of nuclei in syncytial knots. Once the nuclei are aggregated into knots apoptosis if finally allowed to complete (91). This hypothesis is very controversial and several papers with evidence both supporting and in the contrary have been published in recent years. For supportive data Gauster et al. (2009) found that an antibody against activated caspase-8 was localized to 0.24% of CT in placental cross sections (95). With this data the authors concluded that these were the proportion of CT actively undergoing fusion. These conclusions are based almost entirely on the data in Black et al. (2004) for no evidence of intercellular fusion is presented in the manuscript (90). Data refuting the importance of activated caspase-8 was presented in a paper by Guilbert et al. (2010) (87). In this paper the authors used both broad-spectrum caspase inhibitors (ZVAD-fmk) and caspase-8 specific inhibitors (zIETD-fmk, qVD-OPh) on primary CT and explants. They observed no inhibition of fusion in either model and concluded that trophoblast fusion does not require the active form of caspase-8. This does not exclude the involvement of pro-caspase-8 in trophoblast differentiation, for this form of the protein has been shown to be involved in T and B-cell proliferation and monocyte differentiation (88).

1.4.2.1.7 Positive and Negative Regulators of Fusion Competence

It is important to remember, as already stated above, that multiple growth factors and hormones as well as the generation or exogenous addition of

second messengers (cAMP) lead to acquisition of fusion competence and ultimately fusion *in vitro.* Thus regulation of the acquisition of fusion competence undoubtedly involves the integration of both pro- and anti-differentiation signals. Though the list of pro-differentiation molecules continues to grow, anti-differentiation agents, those that actually inhibit differentiation, are limited to known apoptosis-inducing factors such as TNF-alpha and interferon gamma (96). These negative signals are important areas for research in the future for removal of the negative signals inhibiting differentiation are likely to be equally as important in the overall process as positive stimulation.

1.4.2.2 Fusion and Migration

A second specific stage of trophoblast differentiation that is likely to occur requires bringing the membranes that will fuse in close approximation to each other. In the first trimester placenta when the CT layer is continuous this is unlikely to involve migration on the part of the CT, for it is much more likely that the CT directly in contact with the fusion competent section of ST will also be activated to fuse. In the third trimester this step is much more likely to involve CT migration. The molecular signals guiding this directional migration are currently completely unknown, and indeed that this is actually a required step in trophoblast differentiation remains to be proven experimentally. It is likely that this step would occur concurrently with the acquisition of fusion competence.

1.4.2.3 Membrane Fusion- Phosphatidylserine Externalization

The key step in trophoblast differentiation is the actual fusion of the CT and ST membranes. Externalization of the phosphatidylserine (PS), a phospholipid that is normally found on the inner-leaflet of the plasma membrane has previously been shown to be required for the actual fusion of trophoblastic membranes (4,97). This work was completed exclusively using choriocarcinoma cell lines and has never been repeated with primary cells. This remains the only work elucidating mechanistic details about the cellular mechanisms of fusion. The externalization of PS is a hallmark of early apoptosis and this observation has also been used as evidence that the initiation of apoptosis is required for trophoblast differentiation. PS externalization independent of apoptosis has been shown to be important for the fusion of myoblasts though, so linkage to the apoptosis hypothesis is weak (98-100).

1.4.2.4 Functional Differentiation

The final step of differentiation is the functional incorporation of the CT into the ST. As stated above limited numbers of CT have been shown by electron microscopy to have intracellular organelle constituents that more closely resemble the ST than sister CT so functional differentiation is likely to begin before fusion of the cells. Since functional differentiation may occur concurrently with fusion it is difficult to elucidate specific signals that stimulate each process. Models such as EGF stimulation of primary CT will be useful to further understand the independent molecular control of fusion

versus functional differentiation for this model has been shown to stimulate fusion but does not necessarily result in the production of hCG (64). Wice et al. (1990) also observed in Bewo that fusion could be stimulated with theophylline (a phosphodiesterase inhibitor) but did not result in the production of hCG (56). Functional differentiation, like fusion, likely involves many steps. Initially pro-differentiation signals likely stimulate alterations in the organelle composition such as a switch towards production of rough versus smooth endoplasmic reticulum and increased mitochondrial biogenesis producing the CT mentioned above that have a distinctly ST like organelle composition. Since hCG and hPL production are never observed in CT the true functional capability of the cell to function like a ST is somehow controlled until incorporation of the organelles into the syncytium but the mechanisms controlling this are completely unknown (101). Another aspect of functional differentiation requiring study is the trafficking of organelles within the ST. What are the molecular cues and machinery involved in the clustering of rough endoplasmic reticulum, nuclei, or other organelles within the ST once the CT has fused? This question too remains completely unstudied.

1.4.3 Cellular Control of Trophoblast Differentiation:

It is currently completely unclear which cell types initiate trophoblast differentiation. The ST is a likely candidate for master and commander of the process, for it is feasible that when areas of the ST start to lack specific organelles and functional nuclei that trophoblast differentiation is stimulated

by the syncytium itself. Another popular hypothesis is that the expansion of the endothelium stimulates trophoblast differentiation. This theory relies on the notion that capillary growth would outstrip the size of the villi and that the extrusion of the microvasculature into the ST and the mechanical stretch of this situation could stimulate trophoblast expansion (7). Very recently isolated stromal cells have also been shown to secrete activin-A which is able to stimulate trophoblast fusion and thus stromal cells may also play a role in controlling the overall size of the villi (37). It is unlikely that a single population of cells controls trophoblast differentiation and it is much more likely that the balance of pro- and anti- differentiation signals from multiple cell types received by both CT and ST controls the process overall.

One thing that is clear in this process is that trophoblast differentiation and the extension of the placental blood vessels, or angiogenesis, are inextricably linked for villous sprouts are very quickly invaded by expanding endothelium and avascular villi are almost never observed in normal placentas.

1.4.4 Angiogenesis

Placental angiogenesis, or the extension of existing blood vessels, is the second process that is required for placental growth. Vasculogenesis, the formation of *de novo* blood vessels, begins in the placenta at 21 days post coitus and from this point on vasculature is at the core of every villi (1,102). The developing capillary network does not connect to the primitive umbilical cord until 32 days post coitus and from this point on flow is initiated and the

placental vasculature carries oxygen, waste, and nutrients between the mother and the fetus (102). Like all vascular networks the placental vasculature is a connected set of trees with branching leading to progressively smaller caliber vessels, but in comparison to the lung, by the end of gestation the placenta is relatively poorly branched (102). From week 7 to 25 of pregnancy the placental vasculature is much more highly branched than at term with branching regressing to become longer, thinner, and less-branched networks after 25 weeks (102). The highly branched arrangement of the vasculature before 25 weeks is present when there are still two complete layers of trophoblasts between the fetal and maternal circulation therefore it could be postulated that increased branching is required to somehow overcome the larger diffusion distance for oxygen of this arrangement, with the remodeling to a less branched form over the remaining half of gestation representing the lessening of the distance between the fetal and maternal blood.

It has long been thought that the proliferative angiogenesis that occurs after 25 weeks results in hyper-elongated villous capillaries that loop and obtrude on the overlying trophoblastic layer. This obtrusion is thought to result in blister like areas in the ST and to stimulate the formation of new terminal villi (102). Recently it has been shown that terminal villous formation can also occur from blind ended capillaries, implying that branching angiogenesis continues to play an important role in placental growth after 25 weeks (7)

and that the previously stated rule that branching angiogenesis ceases in the later half of gestation is likely to be untrue.

1.4.4.1 Mechanisms of Placental Angiogenesis

The mechanisms stimulating placental angiogenesis are largely unknown at this point and the cells involved in the process can only really be hypothesized based on data about the expression pattern of various angiogenic growth factors throughout gestation. One of the key regulators of angiogenesis is vascular endothelial growth factor (VEGF). VEGF is responsible for stimulating branching angiogenesis and has been shown, using knockout models, to be absolutely necessary for development of the vasculature (103). In the first trimester VEGF has been shown to be expressed by CT and Hofbauer cells by immunohistochemistry with expression shifting towards the ST and stromal cells later in gestation (51,102). Another key regulator of angiogenesis is angiopoeitin-2 (Ang-2). This growth factor plays a role in vascular stabilization and through its receptor Tie-2 promotes a loosening of the perivascular cells and of the adhesion of endothelial cells to one another priming the vasculature for remodeling (104). Ang-2 is expressed in the first trimester by the cytotrophoblasts, but by term expression is restricted to the stromal cells (41). The placenta has also been shown to express other pro-angiogenic growth factors including: placental growth factor (PlGF), hepatocyte growth factor (HGF), basic-fibroblast growth factor (b-FGF), endocrine-gland vascular endothelial growth factor (EG-VEGF), and angiogenin (40,43,105-

107). These factors have all been shown by immunohistochemistry to localize to the fibroblastic stroma and/or endothelium and the trophoblasts. The placenta is also a rich source of anti-angiogenic growth factors such as: soluble Flt-1 (sFlt), pigment epithelial derived factor (PEDF/Serpin F1), and the glycoprotein thrombospondin-1 (108-110) with expression again being primarily by the fibroblastic stroma, endothelium, and trophoblasts.

1.4.4.2 Cellular Regulators of Placental Angiogenesis

The CT and ST are primary sources of large amounts of both pro- and anti-angiogenic factors and are undoubtedly involved in the regulation of villous angiogenesis, but all of the other cellular components of the villi also express various growth factors at different points in gestation (102,111). Essentially no data are available on the individual contributions and relative importance of the different villous cell types to placental angiogenesis, though clues as to the roles different cells play in the process can be found in the cancer and developmental angiogenesis literature. Hofbauer cells are likely to be key players in early vasculogenesis and angiogenesis for in addition to expressing VEGF they also express the Ang-2 receptor Tie-2 (52). Tie-2 expressing monocytes have been shown in cancer to play important paracrine roles in the development of tumour vasculature (112) and in development Tie-2 expressing tissue macrophages were shown to be directly physically involved in the anastomosis of new vessels in mice (53). In addition, fibroblasts have also been shown to be important for angiogenesis in both tumors and wound healing sites through the production and degradation of extracellular matrix

and the production of growth factors and are thus very likely to play similar roles in the placenta (113,114). A third population of cells that are known to be very important in the regulation of angiogenesis are pericytes. The precise role of pericytes in angiogenesis remains to be elucidated, but pericyte recruitment to the basement membrane of newly formed endothelial cell tubules seems to be required for stabilization and maturation of new vascular sprouts. Additionally loosening of the adhesion of pericytes to the endothelial basement membrane is required for endothelial sprouting to occur (48). Pericyte coverage is predicted to control the plasticity of a vascular bed and the pericyte-free endothelial tubules of the retina are controlled much more by soluble VEGF concentrations than other vascular beds where pericytes are present (48). Pericyte coverage of the placental vasculature is variable and it has been shown that less than 20% of the vasculature that is under 30μm in diameter have pericyte coverage (49). Thus the absence or presence of pericytes within the placental vasculature likely plays important roles in the control of vascular plasticity.

1.4.4.3 Hypoxia and Angiogenesis

Micro-environmental oxygen concentrations are also likely key regulators of placental angiogenesis. VEGF, PlGF, and angiopoietin expression are all influenced by the local availability of oxygen through transcriptional regulation and/or mRNA stability and there is experimental evidence in both trophoblasts and placental fibroblasts that oxygen can control the expression of these key angiogenic mediators (4,115). Hypoxia is a known stimulator of

angiogenesis through the up-regulation of pro-angiogenic factors such as VEGF and Ang-2 and as in all other vascular beds is undoubtedly a key regulator of placental angiogenesis (104).

1.5 Intrauterine Growth Restriction (IUGR)

Intrauterine growth restriction (IUGR) is a common pregnancy complication defined as the failure of a fetus to reach its genetically predetermined growth potential for a given gestational age (116,117). IUGR has several etiologies, with the condition arising due to maternal, fetal, placental, or external factors and affects ~10% of all pregnancies (116,117). IUGR is most commonly due to placental causes, and is often labeled as placental insufficiency, though other major causes include fetal chromosomal abnormalities (chromosomes 18, 13, and 21), fetal and maternal infection (toxoplasmosis, rubella, cytomegalovirus, human immunodeficiency virus, malaria), and maternal under-nutrition, substance abuse or smoking (1,55,117).

As stated above IUGR infants have reduced placental weight when compared to appropriate weight for gestational age infants (54) and IUGR has been shown to be associated with various malformations and more subtle villous maldevelopment (1,117). IUGR can develop any time beyond 20 weeks but in the clinic the majority of cases observed are classified as term IUGR (117). In this presentation the fetus grows appropriately until the final weeks of pregnancy resulting in term deliveries of very small babies. The more uncommon presentation is preterm IUGR and this manifestation is associated with increased perinatal morbidity and mortality compared to term IUGR

(118). Thus much of the data on villous maldevelopment focuses on preterm IUGR for these pregnancies are much more likely to have negative outcomes. Irrespective of the timing of onset IUGR is associated with increased fetal morbidity and mortality and babies born IUGR have an increased risk of cardiovascular disease as adults, thus representing a lifelong burden to the health care system (55,117,119).

1.5.1 Villous Maldevelopment

The primary site of villous maldevelopment that has been observed in preterm IUGR is the terminal villi. A significant reduction in the volume of this key exchange interface has been observed using stereological examination (120). Additionally, the surface of preterm IUGR placentas that have absent or reversed end diastolic flow in the umbilical artery (ARED) have been shown to have hugely reduced branching, appear extremely elongated compared to normal gestational age matched controls, and have increased stromal deposition of collagen and laminin (121,122). This morphological data correlates well with the observation that the preterm IUGR fetus (especially those with ARED) are often hypoxic and acidotic, for the terminal villi are the primary site of gas exchange between the mother and the fetus (117).

1.5.2 Etiology of IUGR- Hypoxia

The prevailing hypothesis on the cause of placental maldevelopment in IUGR centers on the deficient conversion of the uterine spiral arteries, the vascular

bed directly responsible for delivery of maternal blood to the intervillous space. In normal pregnancy the physiologic conversion of the spiral arteries into a high capacity low resistance vessel occurs primarily in the first and early second trimester and involves the dilatation of the arterial lumen, EVT invasion of the vessel wall, replacement of the endothelium with EVT, and replacement of the elastic media of the vessel with fibrinoid material (123). Importantly, deficient conversion of the spiral arteries has been observed in IUGR and preeclampsia (124). This is hypothesized to decrease blood supply to the placenta resulting in fetal-placental hypoxia and the altered placental structure observed is hypothesized to result from development in this sub-optimal environment.

It is accepted in the literature that during the first trimester the placenta and fetus develop in conditions of very low oxygen (between 15 and 18.5 mmHg) (125). This is because maternal blood flow to the intervillous space is not initiated until the end of the first trimester when the trophoblastic plugs that block blood flow through the spiral arteries are lost (123,125). The low oxygen environment is likely to be important for proper placental formation for low oxygen availability strongly stimulates angiogenesis and has been shown to stimulate cytotrophoblast proliferation both *in vitro* and *in vivo* (125). Thus it is hypothesized that it is not necessarily hypoxia that results in placental maldevelopment in IUGR, but that it is the failure of oxygen tensions to rise in the second trimester and the sustained low oxygen

environment that may be responsible for the observed changes in villous structure (119,125). The increased oxygen tension associated with the removal of the trophoblastic plugs is hypothesized to be a key regulatory event that allows for altered expression of regulatory proteins within the developing villi and results in proper maturation. Whether intervillous hypoxia is indeed present in IUGR is somewhat controversial for decreases in intervillous oxygen levels have never been quantitatively shown. Additionally, in other naturally hypoxic environments, such as high altitude, the placenta has been shown to be remarkably adaptable and the capillary profile has actually been shown to increase indicating a compensatory angiogenesis mechanism to overcome the lower availability of oxygen in the maternal blood (49,126). Evidence such as this does not necessarily refute the existence of long-term severe hypoxia in IUGR though and instead indicates that IUGR may be due to an improper adaptation to a low oxygen environment. Another recent hypothesis is that it is not necessarily the reduction in oxygen that is harmful to the placenta but instead that fluctuations in intervillous oxygen content may be responsible for the placental pathology. This hypothesis relies on the idea that spiral arteries that were improperly remodeled and retain their smooth muscle cell layer would spontaneously constrict resulting in temporarily decreased blood flow to the placenta (125). There is very little evidence supporting this hypothesis though, with most of the evidence being drawn from normal laboring placentas and *in vitro* explants (125).

1.5.3 Etiology of IUGR- Placental Transport

Another important aspect of trophoblast biology that is altered in IUGR is ST transport. The ST is a transport epithelium expressing a wide array of transporter proteins. Adequate transport of nutrients to the fetus is essential for proper fetal growth and the fetus depends on several nutrients that must be maintained at higher levels in the fetal circulation than in the maternal circulation (e.g. amino acids, lactate) (127). Additionally it is known that IUGR fetuses have reduced levels of amino acids and glucose in their circulation (128). IUGR is associated with decreases in the expression of transporters for the amino acids alanine, glycine, and leucine (system A and leucine transporters) as well as Na^+/K^+-ATPase, Na^+/H^+ exchanger, and the free-fatty acid transporter lipoprotein lipase. Hypoxia has been shown to induce a down-regulation in the expression of system-A transporter in cultured trophoblasts (128,129).

1.5.4 Villous Malformation in IUGR: Preserved versus Absent or Reversed Umbilical Artery End Diastolic Flow

Villous malformation is not uniform amongst IUGR samples. As previously stated IUGR+ARED results in a significant reduction in the volume of the terminal villi and a huge reduction in branching, but in preterm IUGR where umbilical artery flow is preserved (PED) an increase in terminal vascular branching is instead observed. It has previously been hypothesized that these two very different vascular arrangements are due to opposing environments

at the level of the terminal villi. Due to the excessively branched structure observed in IUGR+PED it is thought that this represents a situation where both the intervillous space as well as the placenta are hypoxic. Thus the pre-placental and placental hypoxia would stimulate a compensatory branching angiogenesis but this compensation is inadequate and the fetus remains hypoxic (130). In contrast this hypothesis holds that in IUGR+ARED there is pre-placental hypoxia in the intervillous space, but that the placenta itself is hyperoxic resulting in the drastic reduction in branching observed and an impaired delivery of oxygen to the fetus. This hypothesis is very circular in nature though for there is no indication why or how the supposed placenta hypoxia versus hyperoxia would occur and how it would then influence villous structure. Instead it could be predicted that both placentas develop in similarly hypoxic environments and they instead react differently to this hypoxia. When considered in this way ARED fetuses may completely lack the ability to stimulate compensatory angiogenesis due to either different micro-environmental conditions or an inherent inability in any of the key placental cells that are involved in angiogenesis, whereas IUGR+PED retain the ability to at least partially stimulate compensatory angiogenic mechanisms.

1.5.5 Etiology of IUGR- Villous Trophoblast Dysfunction

In addition to the angiogenic malformations and changes in transport function found in IUGR there is also well- documented alterations in trophoblast function and maintenance. IUGR has been associated with a decreased number of CT, an increased number of syncytial knots, and

increased apoptosis in the ST (131-133). These observations are interpreted jointly to indicate a dysfunction in the maintenance of the ST, for if there is a reduced pool of CT from which to differentiate and an increase in the rate of turnover of ST nuclei it is likely that expansion of the ST would be limited. *In vitro* experiments have shown that hypoxia (culture at <1% O_2) leads to an increase in apoptosis in both CT and ST (134). Hypoxia induces apoptosis in the two cell types through the same apoptotic pathway with levels of p53, a key regulator of apoptosis initiation, increasing in CT but decreasing in ST (135). In the ST the pro-apoptotic protein Bad is also increased. p53 levels are also elevated in IUGR, primarily due to ST expression, thus the *in vivo* and *in vitro* situations appear to be similar with hypoxia appearing to have the capacity to induce the *in vivo* situation (131). *In vitro* experiments utilizing explant cultures have also shown an increase in syncytial knot formation due to hypoxia, hyperoxia, and the presence of reactive oxygen species (19).

1.6 General Objectives and Hypotheses

Hopefully the above introduction has impressed upon you just how little is ultimately known about the control of placental growth overall. For an organ with such immense importance and a relatively high burden of disease directly resulting from placental problems, precious little is known in order to develop effective treatments. Thus the overall objective of this thesis is to examine aspects of both of the key regulatory steps in placental growth: trophoblast differentiation and angiogenesis. The first half of this thesis will present studies challenging the hypothesis that the initiation of apoptosis is involved in trophoblast differentiation. Specifically we examined the role of the caspase-independent mediator of apoptosis, apoptosis-inducing factor (AIF), in trophoblast differentiation. Since the externalization of PS during trophoblast differentiation has also been used repeatedly as evidence of the involvement of apoptosis during trophoblast differentiation, but this event has only been examine in trophoblastic cell lines we also undertook studies to examine whether PS externalization occurs in primary trophoblasts during differentiation.

The second half of the thesis focuses on the second key regulatory step in placental growth: angiogenesis. In this work we were interested in elucidating the contribution of individual cellular constituents of the placenta

to angiogenesis, especially focusing on the villous stroma. We hypothesized that stromal cells would be strongly angiogenic and the third chapter of the thesis presents the identification of a novel population of stromal cells and their contribution to angiogenesis. Having identified a novel population of angiogenic cells within the placental stroma we went on to query whether inherent deficiencies existed in the ability of these cells from IUGR placentas to support angiogenesis. Additionally we were interested to examine whether chronic hypoxia may be contributing to the aberrant angiogenesis observed in IUGR. We therefore examined whether chronic hypoxia could decrease the ability of both normal and IUGR cells to stimulate angiogenesis hypothesizing that maintenance in chronic hypoxia would result in a decreased ability of the cells to stimulate angiogenesis.

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Chapter 2: The Role of Apoptosis-Inducing Factor (AIF) in Trophoblast Differentiation[1]

2.1 Introduction

As addressed in Chapter 1, the ST is a large multinucleate cell in the villous placenta. It forms the most external layer of the barrier between the mother and fetus and is the site of nutrient, gas, and waste exchange. The ST is non-proliferative and is maintained exclusively through differentiation of its stem cell, the CT. There are two steps to CT differentiation: 1) fusion of the CT with the overlying ST and; 2) functional differentiation of the CT to become a functioning part of the ST (1). ST expansion is one of the key processes controlling the overall growth of the placenta therefore elucidation of the mechanisms that control differentiation will potentially allow for development of targeted treatments for conditions such as IUGR in the future.

Currently, the conditions required to stimulate CT differentiation remain to be fully elucidated but it has previously been shown that CT fusion requires

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caspase 8 protease activation and the externalization of PS from the inner to the outer membrane leaflet (2-9). Jointly this work has been interpreted to indicate that the initiation of the caspase-dependent apoptotic cascade is a requirement for the differentiation and fusion of the CT (2,4,10). The importance of caspase 8 activation in trophoblast differentiation has been challenged in several publications where trophoblast fusion and CT differentiation has been observed in the presence of caspase 8 protease activity inhibitors (10,11).

The hypothesis linking trophoblast fusion and the initiation of the apoptotic pathway relies heavily on anti-PS monoclonal antibody-blocking experiments which showed PS externalization to the outside of the plasma membrane is essential for trophoblast fusion (9,12). Aminophospholipids such as PS are normally maintained in the inner leaflet of the plasma membrane and this localization is actively maintained by aminophospholipid translocase (13). PS externalization is a hallmark of early apoptosis but is also known to be associated with non-apoptotic cellular fusion in myoblast to myotubule differentiation (14,15). The cellular signals that result in PS externalization in both apoptosis and myotubule formation remain to be completely elucidated but mitochondrial-mediated release of apoptogenic molecules such as apoptosis-inducing factor (AIF) and cytochrome c are known to be involved, at least in the Jurkat T cell line (16).

Caspase-independent apoptosis is mediated by the translocation of the mitochondrial flavoprotein AIF to the nucleus (17,18). This protein is normally found in the mitochondrial intermembrane space and upon apoptotic execution is released into the cytoplasm and translocates to the nucleus where it causes chromatin condensation and apoptosis. Caspase-independent apoptosis is also known to result in PS externalization in some cell lines (16,19). Cleavage of AIF from the mitochondrial inner membrane has been attributed to both cathepsin B and calpain I and translocation has been effectively blocked using chemical inhibitors of these proteases (20). However the localization and ability of AIF to induce apoptosis has never been examined in trophoblasts.

2.2 Objectives

With evidence mounting that activation of the caspase cascade may not be involved in the apoptotic-like features associated with trophoblast differentiation we felt it a logical extension to examine whether caspase-independent apoptosis may play a role in trophoblast differentiation. Thus in this chapter we examined whether the caspase-independent apoptotic mediator AIF is involved in trophoblast differentiation. To test our hypothesis we utilized an *in vitro* model of isolated primary CT cells stimulated to differentiate with the cell permeant cAMP analog Br-cAMP to model trophoblast fusion and differentiation.

2.3 Methods

2.3.1 Primary Trophoblast Isolation

Primary trophoblasts were isolated from normal term placentas at 37-39 weeks gestation that were delivered by caesarean section without labour after patient consent and full ethics review by the University of Alberta Ethics Committee, Edmonton. The tissue arrived in the laboratory and isolation procedures were started within 30 minutes of delivery. Purified villous CT were isolated by trypsin-DNAse digestion of minced chorionic tissue and immunoabsorbtion onto immunoglobulin (Ig)-coated glass bead columns as initially described by (21) and further developed by (22). After arrival in the laboratory placentas were trimmed of their membranes and cotyledons were removed and thoroughly washed in PBS+ 2% fetal calf serum (FCS, Gibco, Grand Island, NY) until visible blood was removed. Tissue was then scraped from the vasculature and the minced non-vascular tissue was again rinsed in PBS+2% FCS through a metal sieve (40 mesh; Sigma). Tissue digestion was then completed using a digestion solution (2.5 μg/mL trypsin (Gibco catalog # 272750-018), 0.1 μg/mL DNAse (Sigma)) in Locke Ringer's solution (0.154 M NaCl, 5.63mM KCl, 2.38mM $NaHCO_3$, 8.33mM D-glucose; pH 7.8)) at a ratio of 1g tissue to 1mL of digestion solution and incubated at 37^{o}C. Tissue digestion was completed seven times with the initial two rounds discarded and in the subsequent 5 rounds the free trypsinized cells were pooled in PBS+2% FCS on ice. After trypsinization red blood cells were lysed by

incubation with lysis buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM disodium EDTA) for 8 min at room temperature. Cells were then pelleted and washed with PBS+2% FCS and incubated in 10% normal goat serum (NGS) for 20 min to block non-specific binding. Cells were then incubated in antibody mix (CD9 (clone 50H.19, house preparation); major histocompatibility complex (MHC) class I (clone W6/32, Harlan Sera-Lab, Crawley Down, Sussex, UK); MHC class II (clone 7H3, house preparation)) for 45 min on ice. These antibodies were selected for immunoelimination because CT do not express MHC class I, MHC class II or CD9 where as all other nucleated cells express MHC class I, immune cells express MHC class II, and placental stromal cells express CD9, thus all other cells that are present in the placenta or remaining maternal blood are held on the columns while the CT flow through (21,22). They were then pelleted and re-suspended in PBS+2% FCS at a concentration of ~$100x10^6$ cells/5mL and ~$100x10^6$ cells were run through a goat-anti-mouse IgG coated glass bead column using PBS+2% FCS to maintain a constant flow. Cells that passed through the column were collected pelleted and then cryopreserved in fetal calf serum made 10% in dimethylsulfoxide (DMSO) in liquid nitrogen.

2.3.2 Preparation of Tissue for Sectioning

Villous tissue was prepared for sectioning immediately upon receipt of the sample in the laboratory. Specifically, chorionic villi were dissected out and rinsed three times in PBS+2% FCS to remove maternal blood and the tissue was then cut into pieces weighing ~2mg. A minimum of eight randomly

selected pieces were fixed in 4% paraformaldehyde (pfa) for 1h and then immersed in 30% sucrose (Sigma, Oakville, ON) for 24 hours. The tissue was then frozen in OCT (Sakura Tissue-Tekat, Torrance, CA) at -70^{o}C and 5 µm sections were cut on a Shandon tissue cryostat (Thermo Fisher Scientific, Pittsburg, PA), placed on glass slides and stored at -70^{o}C.

2.3.3 CT Cell Culture

CT were rapidly thawed at 37°C, centrifuged, re-suspended at 10^6 per ml in Iscoves Modified Dulbecco's Medium (IMDM, GIBCO) supplemented with 10% FCS (IMDM-FCS) and antibiotics (end concentration penicillin 100 U/ml, streptomycin 100 mg/ml, Sigma, Oakville, ON) and plated as follows: 100 µL were seeded into 96 well tissue culture dishes (Nunc no. 167008, Gibco) and incubated at 37°C in a fully humidified 8% oxygen and 5% CO_2 atmosphere in a Forma model 3130 incubator (Marietta, OH) for 4 h. The plates were then washed twice with warm IMDM and fed either 100uL of pre-warmed (37°C) IMDM-FCS made 10µM with the cell permeable cAMP analog Br-cAMP (Sigma) or an equal volume of pre-warmed IMDM-FCS (controls). The culture medium was changed every 2 days until harvested. Cultures were also carried out in 96 well glass-bottomed plates (VWR, Mississauga, ON, Canada) for enhanced nuclear morphology resolution. After plating onto tissue culture plastic, <0.01% of the population were mesenchymal cells by vimentin staining as previously described (22).

2.3.4 Assessment of Cellular Fusion: Desmoplakin Staining

Cell fusion was assessed by the number of nuclei enclosed in desmoplakin-stained cellular boundaries as per (23) and as previously described (24). Briefly, cells were fixed with methanol for 10 min at -20^{o}C followed by a PBS wash and incubation for 1 hour with 20% NGS to block non-specific binding. Cells were then incubated with anti-desmoplakin (Sigma) at 10µg/mL in antibody diluent (Dako) overnight at 4^{o}C. Wells were washed three times with PBS and Alexafluor goat anti-mouse Ig 488 (1:200; Invitrogen) was added for 1h at room temperature. This was followed by three more PBS washes and nuclei were visualized with DAPI (3µM; Research Organics, Cleaveland) for 10 min at room temperature. Triplicate images of each well were obtained at 10x magnification with an Olympus IX2-UCB microscope equipped with a Roper Scientific camera and a Sutter Instruments Lambda DG-4 fluorescent lamp (Olympus, Melville, NY). We used Slidebook 3.0 (Carsen, Markham, ON, Canada**)** as capture software and for analysis.

2.3.5 Quantitation of Functional Differentiation: hCG-β ELISA

Cell culture supernatants from triplicate wells were collected after 72 hours in culture, pooled and frozen at -20^{o}C. Supernatants were assessed for hCG-β content with an ELISA kit (DRG Instruments, Marburg, Germany) according to the manufacturer's instructions with standards provided. Duplicate wells were set up for every assessment in the ELISA.

2.3.6 AIF and E-cadherin Immunofluorescent Staining, Digital Microscopy and Image Analysis

Antibodies for E-cadherin and AIF were utilized for indirect immunofluorescent staining of fixed tissue sections. Anti-AIF and anti-E-cadherin were applied to 4% pfa fixed tissue sections after antigen retrieval in antigen retrieval buffer (Vector Laboratories, Burlingame, CA) and boiling in a pressure cooker for 5 min. Non-specific antibody interactions were blocked by incubating with 10% BSA, 20% goat serum and human IgG (627 µg/mL; Sigma) for 1 h at room temperature before adding anti-AIF (3 µg/mL; R&D Systems, Minneapolis) and anti-E-cadherin (BD Transductions Laboratories, Franklin Lake, NJ) or non-specific Ig controls [mouse IgG2a,(R&D Systems); rabbit Ig (Santa Cruz);] at the same concentrations. All antibodies were then incubated at 4^{o}C overnight. After washing three times with 0.1% Tween-20 in PBS (PBST) Alexafluor goat anti-mouse and Alexafluor goat anti-rabbit secondary was added (1:200) for 1h at room temperature. Nuclei were visualized with DAPI as detailed in section 2.3.4. and samples were mounted with Vectashield (Vector Laboratories, Burlington, CA, USA). Optical sections (0.4 µm) were obtained for all images using a Zeiss Axiovert 200M microscope equipped with an X-Cite 120 fluorescence illumination system, an ApoTome optical slicer module, and an AxioCam camera computer-controlled with AxioVision 4.5 (Zeiss, Goetingen, Germany).

2.3.7 Quantification of AIF Nuclear Translocation in Cultured CT

Inhibitors of the proteases cathepsin-B (zFA-fmk; 20μM (Sigma)) and calpain (calpeptin; 10μM (Calbiochem, La Jolla, CA)) which have been shown to be involved in releasing AIF from the mitochondria (20) were dissolved in DMSO and added to IMDM+10%FCS+antibiotics at a dilution of 1/1000 immediately upon plating. This concentration of DMSO was also added to the controls. Medium was changed after 48 h and fresh inhibitors were added. After 72 h plated cells were fixed with 4% pfa for 10 min, permeabilized with 0.05% Triton-X100, and blocked for 1 hour with 10% BSA, 20% goat serum and human IgG (as in section 2.3.6). Primary antibody against AIF (3 μg/mL; R&D Systems) or normal rabbit immunoglobulin were then added, the plates incubated overnight at 4^{o}C, followed by two washes in PBST. Subsequently Alexafluor goat anti-rabbit secondary antibody was applied for 1 h at room temperature (1:200), followed by washes with PBST and PBS. Nuclei were visualized as in section 2.3.4. Samples were mounted with Vectashield and visualized with a Zeiss Axiovert 200 M microscope equipped with an ApoTome optical slicer as described in section 2.3.6. At least triplicate images per well were obtained and nuclei were completely visualized with 0.4 μm optical slices. Images were examined using Axiovision 4.5 to examine the stacked composite image of the slices so the entire nuclei for each cell could be examined. Nuclei were considered negative if no AIF staining was seen in any slice.

2.3.8 Annexin-V labelling of Externalized Phosphatidylserine

Trophoblasts were plated as for AIF translocation experiments above and were treated after a 4h incubation with staurosporine (0.3 μM) or Br-cAMP (10μM) +/- calpeptin (20 μM) or DMSO control. The cells were cultured for 24-72 h before washing with Annexin V binding buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$; pH 7.4) and the addition of Annexin V-FITC (1:50 in Annexin V binding buffer; Lonza, Walkersville, MD) for 1 h at 37^{o}C. The cells were then again washed with Annexin V binding buffer and fixed with 4% pfa for 10 min at room temperature. After washing with PBS the nuclei were counterstained with DAPI (as in section 2.3.4) and visualized as described for desmoplakin immunofluorescent staining (section 2.3.4). Triplicate wells of each treatment were plated for each of two placentas. The proportion of Annexin V positive cells were counted and values from each of the three wells per treatment were averaged.

2.3.9 Statistical Analysis

All data, with the exception of the PS externalization experiments in Figure 2-4, came from at least three sets of experimental data collected from cells or tissues isolated from a minimum of three different placentas. PS experiments were performed twice on cells from two different placentas and analyzed with a Kruksal-Wallis test. One-way ANOVA with a Bonferroni post-hoc test were carried for the rest of the results. Results were considered statistically significant if $p<0.05$. The statistical analysis was carried out using the statistics package in Prism 4.0 software.

2.4 Results

2.4.1 Expression of AIF in trophoblasts of the villous placenta

AIF is commonly expressed in all cells (17,18) but the protein is very strongly expressed in the villous trophoblast (Figure 2-1) where the ST and CT are distinguished by E-cadherin staining. CT are surrounded by E-cadherin and the ST, which expresses only low levels, is not (25) but both express high levels of AIF relative to the rather faint expression seen in the villous stroma. AIF was highly expressed in isolated CT after 4 h of culture and was predominantly localized to the mitochondria (Figure 2-2). When optical sections were taken of the entire nuclei of CT it was found that small amounts of AIF were localized to the nucleus of ~50% of CT after 4 h. When CT were stimulated to differentiate into syncytium with Br-cAMP for 72 h the proportion of nuclei containing small amounts of AIF increased to ~75%. Caspase-independent apoptosis is associated with translocation of large amounts of AIF to the nucleus in multiple cell types (17,18) but translocation of small amounts of AIF like that observed with the isolated trophoblasts has not been characterized in other models.

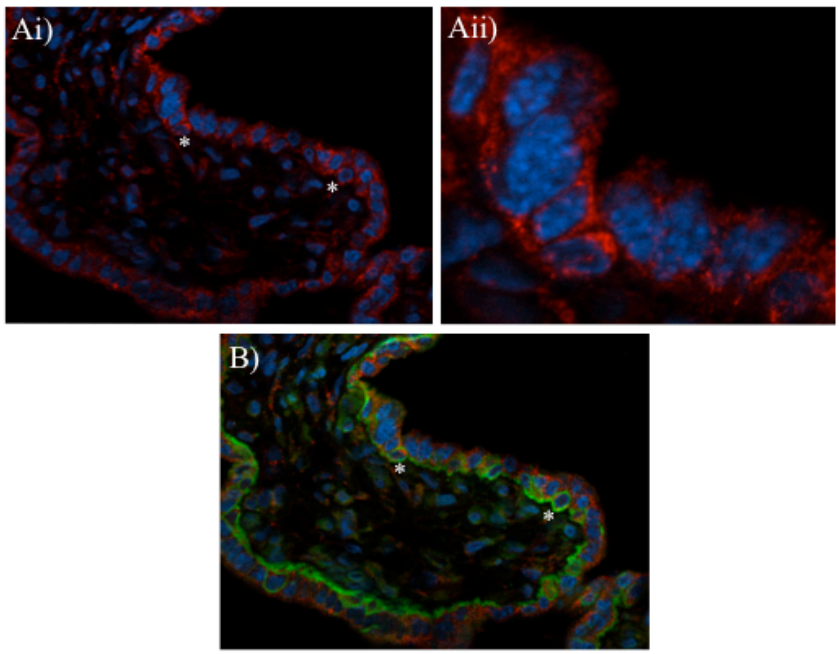

Figure 2-1: Expression of AIF and E-cadherin in the villous placenta
Three color indirect-immunofluorescence staining of AIF (red) and E-cadherin (green) and direct DAPI staining of nuclei (blue) in a 22 wk placenta. Ai) AIF alone; Aii) High magnification image of selected CT and ST nuclei from panel Ai; B) AIF (red), E-cadherin (green); *= E-cadherin-delineated CT. This photomicrograph is typical of >50 taken under the same conditions on three different placentas. Equal concentrations of isotype matched Ig did not stain parallel sections.

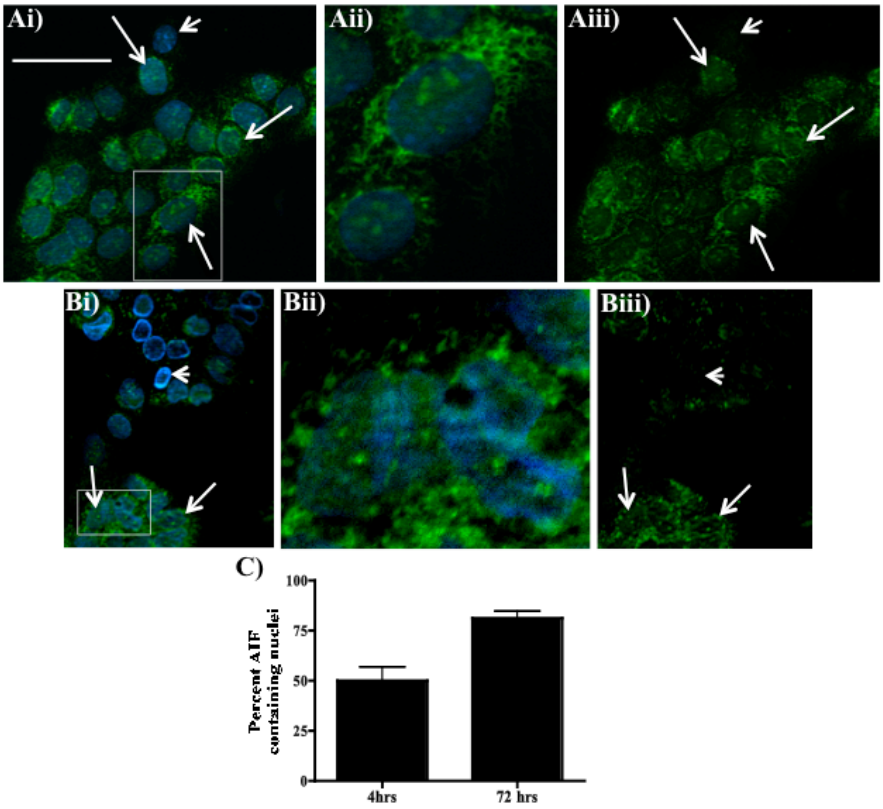

Figure 2-2: AIF translocation from the cytoplasm to the nucleus in primary trophoblasts as a function of Br-cAMP-stimulated differentiation
A) AIF protein present (arrows) or not present (arrowheads) in the nuclei of trophoblasts cultured for 4 h with medium alone. AIF is stained green, nuclei stained blue with DAPI, bar = 25μm for panels A and B. Isotype antibody control staining for AIF was negative (data not shown); Aii) High magnification image of nuclei highlighted with a box from panel Ai where AIF translocation is present; Aiii) AIF staining alone; B): AIF protein in the cytoplasm, mitochondria and nucleus of cells incubated with Br-cAMP for 3 days; C): Percent of nuclei with translocated AIF after culture with medium alone after 4 h and 3 days with Br-cAMP. Depicted are the mean ± SEM in cells isolated from three placentas. All experiments were carried out in an environment of 8% oxygen, 5% CO_2.

2.4.2 Inhibition of AIF translocation does not prevent differentiation

Activation of AIF translocation from the mitochondria to the nucleus can be blocked with cathepsin and calpain inhibitors, respectively zFA-fmk and calpeptin (20). Thus, we next inhibited translocation with inhibitors and determined whether they blocked the translocation of the small amounts of

AIF observed in trophoblasts as well as trophoblast fusion and hCG-β secretion. Calpeptin but not zFA-fmk was able to significantly reduce AIF translocation to the nucleus of trophoblasts (Figure 2-3a) indicating that calpain I but not cathepsin B is likely to be involved in the translocation of AIF in trophoblasts. Neither calpeptin nor zFA-fmk had a significant impact on trophoblast fusion or hCG-β secretion (Figure 2-3b and c). Thus it is unlikely that caspase-independent apoptosis is involved in trophoblast differentiation. Calpeptin treatment also failed to reduce the externalization of PS to the outer membrane of trophoblasts (Figure 2-4). Outer-membrane PS localization, as measured by annexin V, was found on ~5% trophoblasts in primary cells after 72 h in medium alone or Br-cAMP which we have previously found to be approximately the background level of apoptosis in cultured trophoblasts (26).

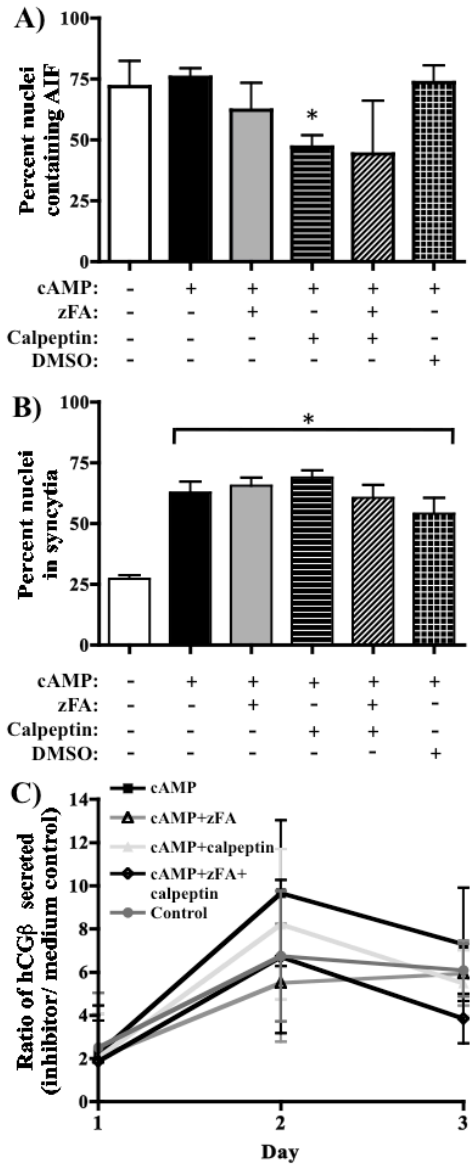

Figure 2-3: Inhibition of AIF translocation fails to block trophoblast differentiation

A) Percent of nuclei containing translocated AIF after treatment with Br-cAMP and cathepsin-B inhibitor zFA or calpain inhibitor calpeptin. DMSO is the solvent control for both inhibitors. B,C) Trophoblast fusion and hCG-β secretion was not inhibited by zFA-fmk or calpeptin after 72 h of treatment with Br-cAMP. The histograms in panels A through C are the average of 3 experiments with CT from 3 different placentas. Bar graphs represent mean ± SEM. Panel C hCG-β secretion is normalized to a medium control containing no Br-cAMP. *=$p<0.05$ versus control; All experiments were carried out in an environment of 8% oxygen, 5% CO_2.

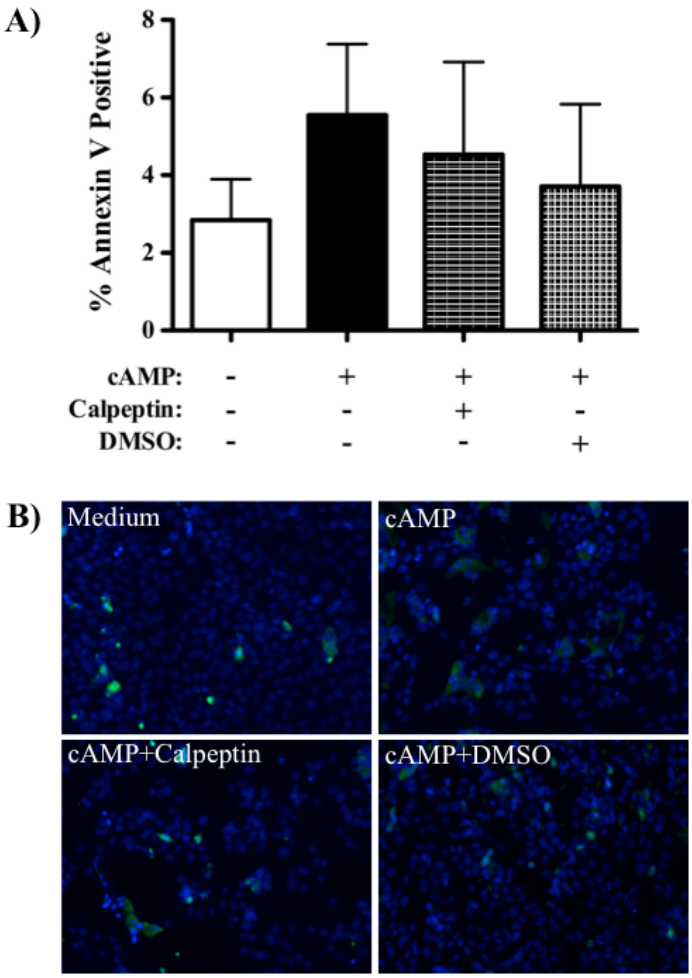

Figure 2-4: Calpeptin fails to decrease the amount of externalized PS
A) Summary graph of annexin-V binding after 72 h; median+/-range; n=2; B) Representative images of annexin-V FITC binding after 72 h. Experiments were carried out in an atmosphere of 20% O_2 5% CO_2.

2.5 Discussion

In this study, we used isolated primary CT stimulated with Br-cAMP as a model of villous CT differentiation. To query whether caspase-independent apoptosis was involved in trophoblast differentiation we examined the cellular localization of the caspase-independent apoptotic mediator AIF. Translocation of AIF from the mitochondria to the nucleus is an essential step in caspase-independent apoptosis (17,18). We found that small amounts of AIF were within the nuclei of a large proportion of CT and ST nuclei but that inhibition of this translocation had no effect on differentiation. These results indicate that caspase-independent apoptosis is unlikely to be involved in trophoblast differentiation.

The hypothesis that CT fusion results from the initiation of the classical caspase-dependent apoptotic cascade (particularly caspase-8) is currently controversial in the field of trophoblast differentiation (3,10,11). We have previously published using the same model of Br-cAMP stimulated isolated primary CT fusion used in this study and found that both broad spectrum caspase inhibitors and caspase 8 specific inhibitors were not capable of blocking trophoblast fusion (11). Thus we were interested to examine whether apoptosis via the caspase-independent mediator AIF could play a role in CT differentiation. The hypothesis on the involvement of early-apoptosis in trophoblast fusion also relies on data that shows that the

externalization of PS is required for trophoblast fusion (10). The mechanisms by which PS-externalization occur in trophoblast differentiation have previously been shown to be ATP and protein kinase A (PKA)- dependent but independent of caspase activation (12). To strengthen our hypothesis that AIF was involved in trophoblast differentiation the translocation of AIF to the nucleus has been shown to temporally coincide with PS-efflux in other cell systems (27). We did not observe PS-efflux above previously reported basal apoptosis levels in primary CT and PS-efflux was not influenced by calpeptin treatment. These results led us to postulate that PS-efflux does not occur to the same degree as previously observed in trophoblastic cell lines and that the small amounts of translocated AIF we observed are not involved in PS-efflux.

The translocation of small amounts of AIF to the nucleus is a novel observation. In other cell systems and *in vivo* tissue where caspase-independent apoptosis is occurring AIF nuclear translocation is massive and is found throughout the nucleus (28,29). This nucleus-filling translocation is what is classically associated with AIF-dependent nuclear condensation and apoptosis (18). It is currently unknown why small amounts of AIF is found in trophoblast nuclei. Br-cAMP treatment for 72 h increased the number of nuclei containing low levels of AIF from approximately 50% to 75% and co-treatment with the calpain inhibitor calpeptin reversed the increase (to

50%). Thus, it appears that at least a portion of the AIF found in trophoblast nuclei relies on calpain cleavage of mitochondrial AIF.

AIF is not, to our knowledge, known to play any roles besides stimulating nuclear condensation through an unknown mechanism while localized to the nucleus. Mitochondrial AIF is involved in stabilization of the electron transport chain and cells with decreased expression of AIF are more susceptible to oxidative stress (30). It is additionally thought to play a major role in mitochondrial metabolism: tissue specific deletion of AIF in muscle or liver results in increased glycolytic rates, insulin hypersensitivity and a resistance to diabetes (30). So though the lethal functions of AIF do not seem to be active during trophoblast differentiation the vital functions of the protein are likely to be important for trophoblast survival. The small amount of nuclear AIF observed in trophoblasts may play a role in cellular metabolism or regulation of trophoblast oxidative stress.

When the results of this study and those of previous studies which have found that caspase activation is not required for trophoblast fusion are considered together we conclude that trophoblast fusion *in vitro* does not involve either known apoptotic cascade. Therefore trophoblast differentiation occurs via an apoptosis-independent mechanism and future research examining the pathways regulating trophoblast differentiation should focus on elucidating the role of non-apoptotic pathways for it is clear

that manipulation both the caspase dependent and caspase-independent apoptotic pathway would be unlikely targets for therapeutic control of differentiation.

A potentially important event during trophoblast differentiation that deserves additional attention by the field is the externalization of PS during fusion. PS appears to be a conserved pathway of cellular fusion amongst the limited types of cells that form multinucleate structures (myoblasts, macrophages, and trophoblasts) thus it may be a key regulatory pathway for cell fusion and an important target for future therapies. A significant secondary observation that we made in this study is that there is very little PS externalization occurring during primary cell differentiation. This is in direct contrast to previous work done with trophoblastic cell lines thus we went on to query in Chapter 3 whether PS externalization is important for primary CT differentiation.

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Chapter 3: Phosphatidylserine externalization is not involved in trophoblast differentiation[2]

3.1 Introduction

The human placenta is a transient organ with immense importance for fetal growth. A properly formed and well functioning placenta is essential for optimal growth of the fetus and aberrant formation and function of the placenta is associated with the common pregnancy conditions of preeclampsia and intrauterine growth restriction (1-3). As introduced in Chapters 1&2, the entire surface of the human placenta is covered by a single giant multinucleate cell, the ST. The ST is a non-proliferative cell type and thus expansion of the ST layer, and therefore the entire placenta, requires differentiation of the ST stem cell, the CT (4,5).

An apparently obligate step that has been identified for trophoblast fusion is the externalization of the membrane phospholipid phosphatidylserine (PS) (6,7). PS externalization has been shown to be involved in fusion of both myoblasts and macrophages with a transient exposure of PS at contact areas

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Contribution: Riddell M.R. was the project coordinator, performed all the experiments and data analyses, wrote the first draft of the manuscript (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.

between two fusing cells (8-10). In trophoblastic cell lines PS externalization was observed to cover essentially the entire surface of fused cells, and persisted after fusion was achieved (6,7,11). Cell fusion was blocked by ~40% in the JAR cell line using one monoclonal anti-PS antibody, though another monoclonal anti-PS antibody had no effect on fusion (6).

PS efflux is an important event in cellular apoptosis, externalization of PS in platelets is involved in the initiation of the clotting cascade, and non-apoptotic PS externalization has been observed in cardiomyocytes (12,13). PS externalization is controlled by the activation of an as of yet unidentified membrane protein or group of membrane proteins known as scramblase(s). Activation of scramblase activity can be stimulated by increases in cytoplasmic Ca^{2+} or apoptotic pathway mediators (though which mediators are involved remains to be identified) (12). In cardiomyocytes PS externalization can also be induced by the inhibition of flippase (13). Flippases are ATP-dependent membrane proteins, such as aminophospholipid translocase, which transport the negatively charged aminophospholipids PS and phosphatidylethanolamine to the inner membrane leaflet and maintain normal membrane asymmetry (12-14). Thus, it is likely the combination of the activation of scramblase(s) and the inhibition of flippase activity that lead to PS exposure on the outer membrane.

3.2 Objectives

As noted previously, PS externalization is considered to be an obligatory event for trophoblast fusion, however this conclusion is based on data produced only in trophoblastic cell lines. Therefore observations obtained using the Bewo model should be validated with primary cells. Additionally, as presented in Chapter 2 Figure 2-4 we found that the levels of PS externalization during primary cell differentiation were very low, thus we sought to examine whether PS externalization was required for fusion in primary trophoblasts.

3.3 Methods

3.3.1 Cells and Tissues

The Bewo choriocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, USA). Primary trophoblasts were isolated from normal term placentas at 37-39 weeks gestation that were delivered by caesarean section without labour after patient consent and full ethics review by the University of Alberta Ethics Committee. Purified villous CT were isolated by trypsin-DNAse digestion of minced chorionic tissue and immunoabsorbtion onto Ig-coated glass bead columns as described in section 2.3.1.

3.3.2 Primary CT Cell Culture

CT were seeded at a density of $1x10^5$ cells per well in 96 well plates in IMDM-FCS and incubated for 4 hours under a fully humidified standard atmosphere. The cells were then washed to remove non-adherent cells and medium was changed to either IMDM-FCS or IMDM-FCS plus 10μM Br-cAMP, 10μM forskolin (Sigma), 10μM 1,9-dideoxyforskolin (Sigma), the combination of Br-cAMP plus 1,9-dideoxyforskolin, or solvent control DMSO in triplicate wells per treatment. Cells were then returned to the incubator for 24, 48, or 72 hours and the medium was changed after 48 hours. Enough cells were plated at once for assessment of annexin-V binding, cell fusion, cytokeratin-18 neo-

epitope staining, and a TUNEL assay after 24, 48, and 72 hours of incubation in parallel wells when cells were treated with Br-cAMP alone.

3.3.3 Bewo Cell Culture

Bewo cells were maintained in Nutrient Mixture F12 Hams (Gibco) supplemented with 15% FCS (Gibco) (Hams-FCS) and antibiotics (end concentration penicillin 100 U/ml, streptomycin 100 μg/ml; Sigma) in 6 well dishes (Corning, Lowell, MA). When 75-85% confluent the cells were passaged using 0.05% trypsin-EDTA (Gibco) for 5 minutes and then seeded into 96 well plates at a density of 10,000 cells per well in Hams-FCS. Cells were incubated for 4 hours in a fully humidified standard atmosphere and medium was changed to Ham-FCS with or without 10 μM forskolin, 10μM 1,9-dideoxyforskolin, 250μM Br-cAMP, both Br-cAMP and 1,9-dideoxyforskolin, or solvent control DMSO. Medium was changed after 48 hours and cells were incubated for a total of 72 hours. Lower concentrations of Br-cAMP were tested (10-100μM) but were not found to stimulate statistically significant rates of fusion (30% fusion or less).

3.3.4 Assessment of Cell Fusion

Primary trophoblasts were fixed after 24, 48, or 72 hours in culture with methanol and stained for the cellular junction protein desmoplakin as described in section 2.3.4.

Bewo cellular fusion was assessed with an E-cadherin antibody. After 72 hours in culture cells were fixed with methanol for 10 minutes followed by a

one hour incubation with 20% NGS and then incubated overnight at 4°C with anti-E-cadherin (5μg/mL; Biolegend; San Diego, CA) or the appropriate non-immune control. The cells were then washed and incubated with Alexafluor goat anti-mouse 488 for one hour and the nuclei were visualized with DAPI for 10 min at room temperature. Triplicate images were captured per well on an Olympus IX2-UCB microscope equipped with a Roper Scientific camera and a Sutter Instruments Lambda DG-4 fluorescent lamp (Olympus, Melville, NY). Slidebook 3.0 (Carsen, Markham, ON, Canada**)** was utilized as capture software and Image-J for analysis. The percentage of cells lacking expression of E-cadherin was assessed, since E-cadherin expression has been shown to decrease with differentiation into syncytium (7).

3.3.5 Annexin-V Binding

Both primary trophoblasts and Bewo were washed once in annexin-V binding buffer (see section 2.3.8) then annexin-V-FITC (Biolegend, San Diego, CA) was added in annexin-V binding buffer (1:50 for primary cells and 1:25 for Bewo) and the cells were incubated for 1 hour in an incubator under standard conditions. Cells were then washed twice with annexin-V binding buffer and fixed with 4% pfa for 10 min. Nuclei were visualized with DAPI and triplicate images of each well were obtained as presented in section 2.3.8. The number of nuclei associated with areas of annexin-V binding were counted. As a positive control primary trophoblasts and Bewo were incubated with 0.3 μM staurosporine for 4 hours and then annexin-V binding was visualized to establish the amount of annexin-V required to visualize PS

externalization. Experiments were carried out on triplicate wells per treatment on cells from a minimum of 3 placentas for primary cells, and on triplicate wells per treatment in 3 separate experiments for Bewo.

3.3.6 Measurement of Apoptosis with Cytokeratin-18 neoepitope

The amount of cell death by apoptosis occurring in primary trophoblasts was assessed by immunofluorescent staining for cytokeratin-18 neoepitope after 24, 48, and 72 hours in culture. Cells were fixed in methanol for 10 minutes at -20^{o}C followed by a PBS wash and then non-specific binding was blocked with 20% NGS for 1 hour at room temperature. Anti-M30 cytodeath (1:30; Roche; Mannheim, Germany) and isotype control mouse IgG_{2b} was incubated overnight at 4^{o}C, followed by repeated washing with PBS. Secondary antibody and DAPI were then applied as presented in section 2.3.4. and images were obtained also as presented in section 2.3.4. The percentage of cells undergoing apoptosis was obtained by counting the number of positive cells.

3.3.7 Measurement of Apoptosis with the TUNEL Assay

The amount of cell death by apoptosis was also assessed with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay after 24, 48, and 72 hours in culture. Cells were fixed with methanol for 10 minutes at -20^{o}C followed by a PBS wash. Cells were then preincubated with TdT buffer (30mM Tris-base, pH 7.2; 140mM sodium cacodylate; 1mM cobalt chloride) for 10 minutes at room temperature. TUNEL reaction (5 µL TdT, 16.5 µM

dATP, 16.5 μM bio-16-dUTP in TdT buffer) was carried out at 37 ^{o}C for 1 hour. The reaction was stopped by adding 2X SSC (300mM NaCl; 30mM sodium citrate) for 10 minutes. Cells were then rinsed with PBS and blocked with 20% NGS for 1 hour at room temperature. Alexafluor 488 Streptavidin (1:200; Invitrogen) was added for 1 hour at room temperature and nuclei were visualized with DAPI as presented in section 2.3.4.

3.3.8 Phosphatidylserine Antibody Neutralization Assay

Medium was changed in primary trophoblasts after 4 hours in culture to medium with or without Br-cAMP (10 μM) and plus either anti-PS antibody (15μg/mL AbCam clone 4B6; Cambridge MA, USA) or control mouse IgG (15μg/mL; Sigma). Medium was then changed every 24 hours. The anti-PS antibody was dialyzed using a Slide-A-Lyzer dialysis cassette (10,000 MWCO; Thermo Scientific) to remove sodium azide. After 72 hours the cells were washed once with PBS, fixed with methanol and stained for desmoplakin presented in section 2.3.4 with the following modifications: after fixation Alexafluor goat-anti mouse 488 (1:100) was added for 30 min followed by a PBS wash and then the normal desmoplakin staining procedure was carried out. Anti-desmoplakin binding was visualized with Alexafluor goat anti-mouse IgG_1-546 (1:200).

3.3.9 Statistical Analysis

All experiments were performed a minimum of three times with experiments utilizing primary trophoblasts carried out on cells isolated from at least 3

different pregnancies. Experiments utilizing Bewo were repeated a minimum of three times. A repeated measures 2-way ANOVA with a Bonferroni post hoc analysis was used to analyze primary trophoblast annexin-V binding, fusion, and appearance of apoptotic markers. A one-way ANOVA with a Bonferroni post hoc analysis was used to analyze the data for fusion and annexin-V binding in the forskolin experiment on both primary trophoblasts and Bewo. A two-way ANOVA with a Bonferroni post hoc analysis was used to analyze the data for the PS antibody neutralization experiments. All statistics were carried out using Prism 5.0 software and a $p<0.05$ was considered significant.

3.4 Results

3.4.1 Primary trophoblast PS externalization and apoptosis

In order to examine whether PS externalization was occurring in primary trophoblasts, cells were probed with annexin-V-FITC. Parallel wells were examined for the apoptotic markers cytokeratin-18 neo-epitope (cyto-18) and TUNEL in order to establish whether PS externalization was occurring at similar rates to apoptosis or rates more similar to fusion. Rates of CT fusion rose significantly over 48 hours when ~30% of all nuclei were incorporated into multinucleate syncytia and Br-cAMP significantly increased fusion after 48 hours (Figure 3-1a). We have previously shown that the spontaneous fusion rate of primary trophoblasts after 72 hours is ~30% and that treatment with Br-cAMP for the same time period leads to fusion of ~75% the cells (15). In parallel wells PS externalization measured with annexin-V peaked after 24 hours in culture at ~3% positive cells and fell over time to ~1% by 72 hours (Figure 3-1b). The appearance of apoptotic markers cyto-18 and TUNEL followed similar patterns to PS externalization with both peaking with ~3% positive cells after 24 hours and falling to <1% after 72 hours (Figure 3-1c, 3-1d).

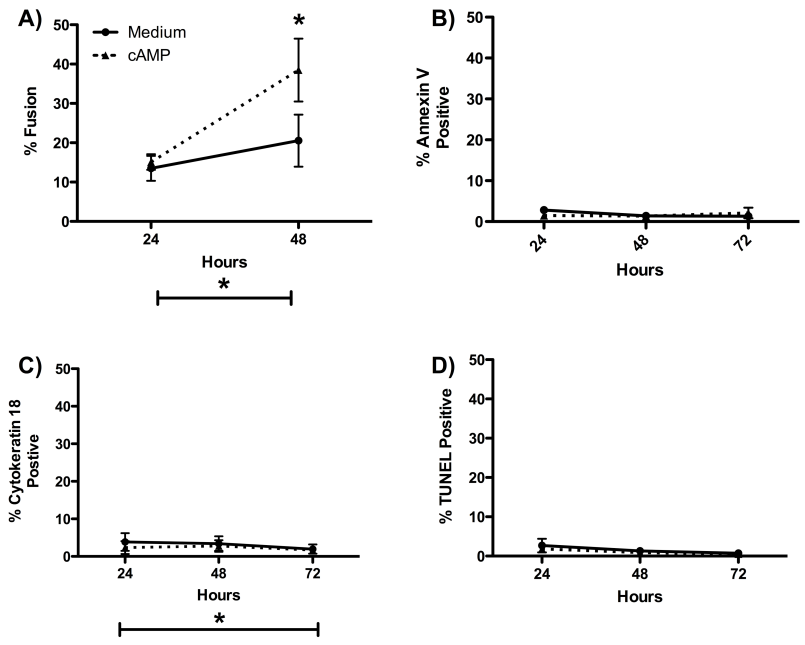

Figure 3-1: Levels of PS externalization more closely resemble apoptosis rates than fusion in primary CT
A) Summary graph of the rate of cellular fusion in primary CT; mean +/- SEM, n=3; repeated measures two-way ANOVA with Bonferroni post hoc analysis; *=p<0.05; B) Summary graph of the proportion of cells with exposed PS measured with annexin-V-FITC analyzed in parallel wells of primary CT to those analyzed for fusion; C) Summary graph of apoptosis measured with cyto-18 in parallel wells of primary CT; *=p<0.05; D) Summary graph of apoptosis measured with TUNEL in parallel wells of primary CT.

3.4.2 The effect of forkolin treatment on PS externalization in trophoblastic cells

In contrast to treatment with Br-cAMP, treatment of primary CT with forskolin for 72 hours led to fusion of ~60% of nuclei concomitant with the same proportion of cells having externalized PS (Figure 3-2a, 3-2b). Since forskolin treatment and addition of Br-cAMP stimulate trophoblast fusion through seemingly similar mechanisms (increased intracellular cAMP concentrations) but result in very different patterns of PS externalization we went on to examine whether this may be due to pleiotropic effects of

forskolin beyond adenylate cyclase activation. Forskolin is known to modulate voltage gated K^+ channels, to inhibit the glucose transporter GLUT1, and interact with ATP-binding cassette sub-family member B1 (ABCB1) in addition to modulating adenylate cyclase activity (16-18). An analog of forskolin, 1,9-dideoxyforskolin, exists which does not stimulate adenylate cyclase activation but interacts with and modulates these membrane transporters (16-18). Thus we utilized 1,9-dideoxyforskolin to understand whether the non-adenylate cyclase effects of forskolin may be causing the observed increased proportion of cells with externalized PS. 1,9-dideoxyforskolin treatment for 72 hours did not significantly

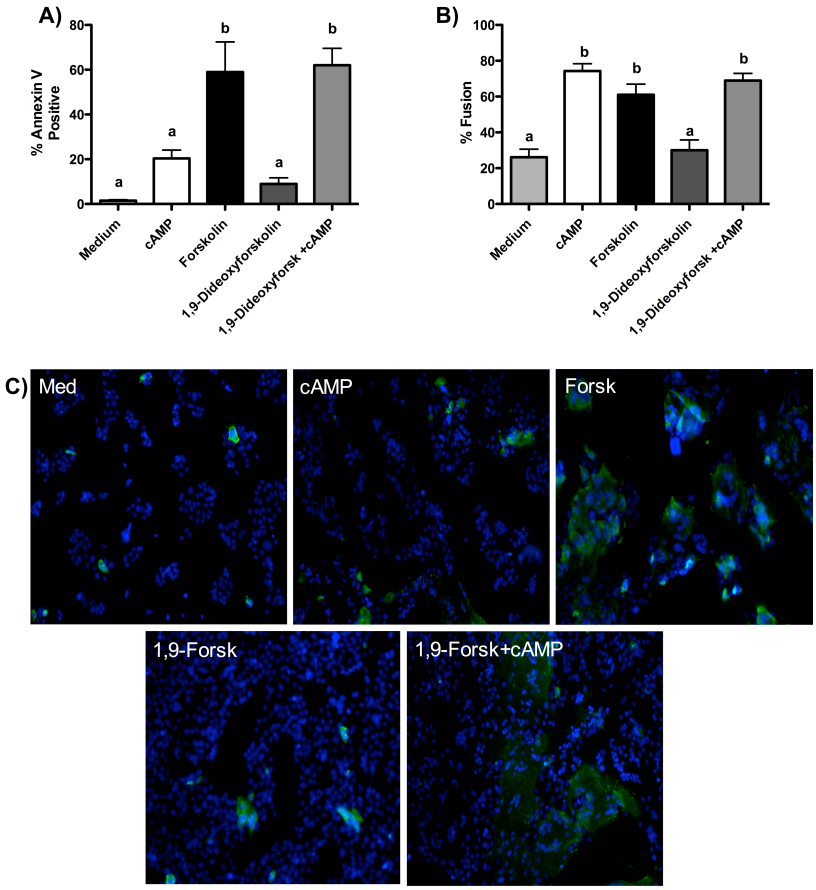

Figure 3-2: PS externalization and cellular fusion in primary trophoblasts treated with forskolin or 1,9-dideoxyforskolin
A) Summary graph of annexin-V positive cells after 72 hours; n=3; mean+/- SEM; one-way ANOVA with Bonferroni post-hoc analysis; a=$p<0.05$ vs medium, cAMP, and 1,9-dideoxyforskolin; B) Summary graph of cellular fusion after 72 hours; n=3; mean +/-SEM; one-way ANOVA with Bonferroni post-hoc analysis; b=$p<0.01$ vs. medium and 1,9-dideoxyforskolin; C) Representative images of annexin-V-FITC binding after 72 hours; Forsk= forskolin; 1,9-Forsk= 1,9-dideoxyforskolin.

stimulate PS externalization or trophoblast fusion (Figure 3-2a, 3-2b; representative fusion images figure 3-3). In combination with Br-cAMP 1,9-dideoxyforskolin stimulated high levels PS externalization (~60% positive nuclei) and trophoblast fusion (~70%) (Figure 3-2a, 3-2b).

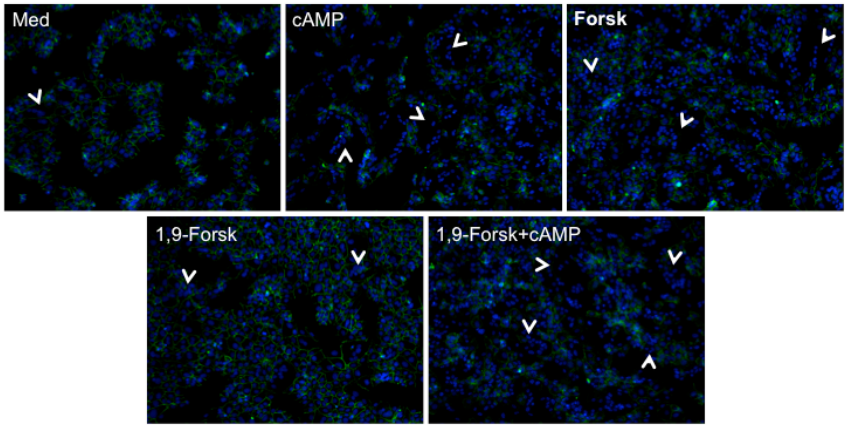

Figure 3-3: Primary trophoblast fusion 72 h after treatment with Br-cAMP and forskolin analogs
Representative images of desmoplakin immunofluorescence; green= desmoplakin; blue= nucleus; Arrowheads indicate multinucleate areas; Med= medium; cAMP= Br-cAMP; Forsk= forskolin; 1,9-Forsk= 1,9-dideoxyforskolin.

Treatment of Bewo choriocarcinoma cells with Br-cAMP stimulated cellular fusion (~70%), but did not stimulate high levels of PS externalization (~4%) (Figure 3-4a, 3-4b and representative fusion images Figure 3-5). Forskolin stimulated widespread cellular fusion (~65%), but PS externalization was limited (~10%) in the cell line, though high variability was observed in the amount of PS externalization observed. Treatment with 1,9-dideoxyforskolin alone did not significantly stimulate cellular fusion or PS externalization. High levels of cellular fusion were observed when Bewo were treated with both Br-cAMP and 1,9-dideoxyforskolin and the proportion of cells with externalized PS rose (to ~10%), but not significantly above medium alone. Again, as with forskolin treatment a very high variability was observed in the proportion of cells with externalized PS with this treatment.

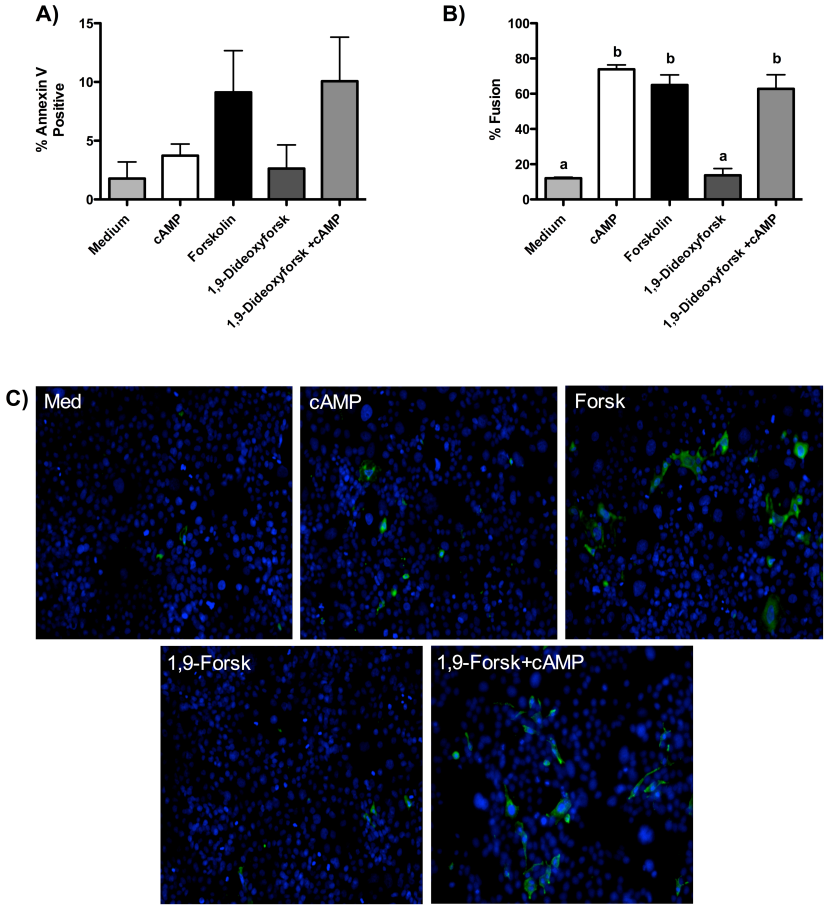

Figure 3-4: Externalized PS levels are low in Bewo despite high amounts of cellular fusion

A) Summary graph of annexin-V positive cells after 72 hours; n=4; mean+/- SEM; one-way ANOVA with Bonferroni post-hoc analysis; B) Summary graph of cellular fusion after 72 hours; n=4; mean +/-SEM; one-way ANOVA with Bonferroni post-hoc analysis; b=$p<0.01$ vs. medium and 1,9-dideoxyforkolin; C) Representative images of annexin-V-FITC binding after 72 hours; Forsk= forskolin; 1,9-Forsk= 1,9-dideoxyforskolin.

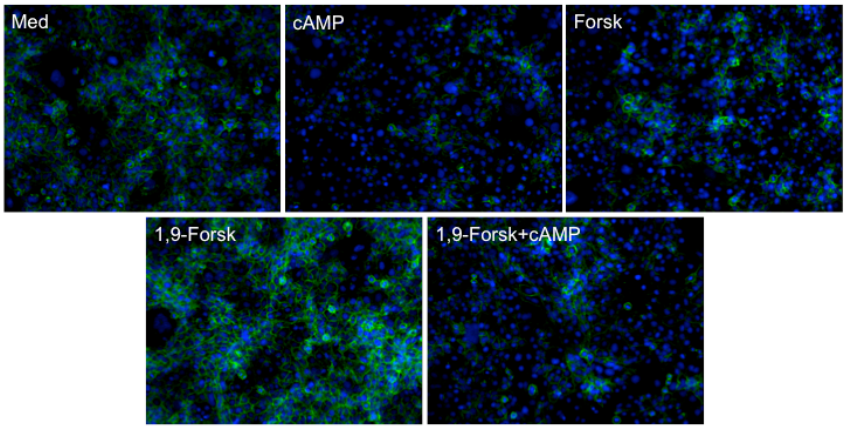

Figure 3-5: Bewo fusion 72 h after treatment with Br-cAMP and forskolin analogs
Representative images of E-cadherin immunofluorescent staining; green= E-cadherin; blue= nucleus; Absence of green staining represents multinucleate areas; Med= medium; cAMP= Br-cAMP; Forsk= forskolin; 1,9-Forsk= 1,9-dideoxyforskolin.

3.4.3 Anti-PS treatment of primary CT throughout differentiation

In order to examine whether neutralization of externalized PS would block primary trophoblast fusion, as was previously shown in the JAR cell line by Adler et al. (1995), primary CT were treated with a monoclonal anti-PS antibody throughout the differentiation process. No differences were observed in the amount of fusion after 72 hours when anti-PS antibody was present (Figure 3-6).

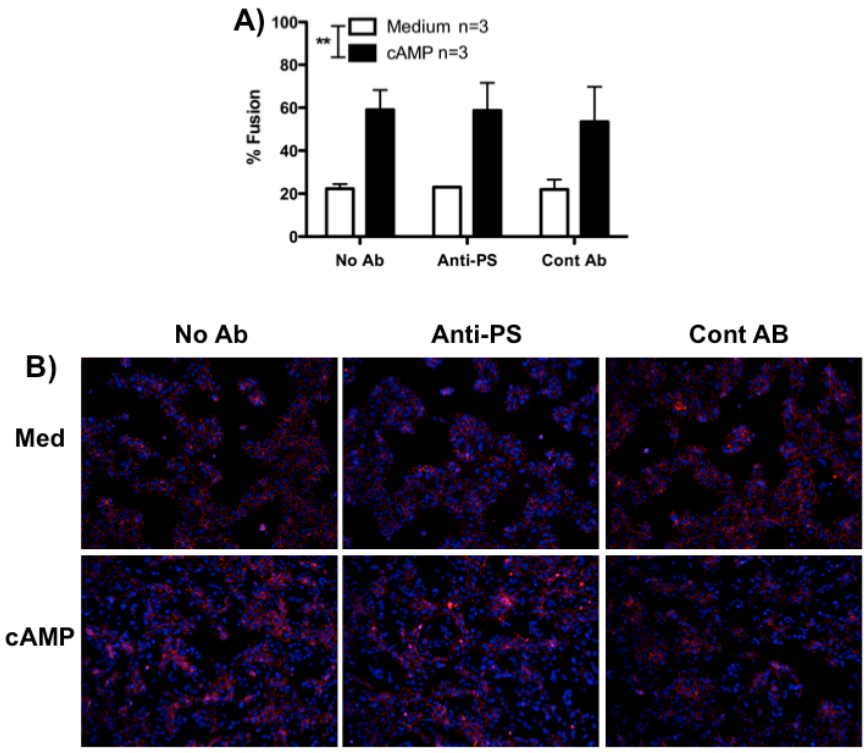

Figure 3-6: Anti-PS antibody fails to block primary trophoblast fusion when present throughout the 72 h differentiation process
A) Summary data of cellular fusion assessed by desmoplakin immunofluorescence; n=3; Two-way ANOVA with Bonferroni post-hoc analysis; *=$p<0.05$; B) Representative images of desmoplakin staining (red) after 72 h of treatment; No Ab= no antibody; Cont Ab= mouse IgG_1 isotype control; med= medium.

3.5 Discussion

In this study we examined whether PS externalization was involved in primary trophoblast differentiation. We found that extensive PS externalization only occurs when differentiation is stimulated with the adenylate cyclase activator forskolin or the combination of Br-cAMP and the forskolin analog 1,9-dideoxyforkolin, and that Br-cAMP treatment alone and spontaneous trophoblast fusion do not result in PS externalization in primary trophoblasts. Therefore extensive PS externalization is likely due to modulation of membrane transporters by forskolin and its analog and is a process that requires increased intracellular cAMP concentrations. Our data also demonstrated that PS externalization is independent of trophoblast fusion in primary trophoblasts.

We also assessed Bewo cells and found that the proportion of cells that were observed to have externalized PS was much lower than the proportion of fused cells observed and that extensive PS externalization was not consistently observed. Previously, using data produced in trophoblastic cell lines stimulated to fuse with forskolin, it was established that PS externalization on the entire surface of syncytium occurred concomitant with cellular fusion and that a monoclonal anti-PS antibody was capable of inhibiting cellular fusion (6,7,11). The authors concluded based on this work

that PS externalization was required for trophoblast fusion. These data are in contrast with our findings but a direct comparison of data are not available as the previous publications that utilized Bewo do not contain a summary of the proportion of cells that are positive for externalized PS and do not present an increase in PS externalization over a medium alone control after forskolin treatment (7,11). Additionally images of positive staining are not presented or limited to clusters of fewer than 10 nuclei, thus making comparison to our experiments difficult (7,11).

Forskolin and 1,9-dideoxyforskolin are known to interact with and modulate activity of voltage gated K^+ channels, the glucose transporter GLUT1, and ABCB1 (MDR1) (16-18). Since the exact transporters responsible for PS externalization remain to be elucidated it is possible that forskolin and 1,9-dideoxyforskolin stimulate one or more of these unidentified transporters directly or cause the inhibition of flippases, which maintain normal phosphlipid membrane asymmetry (12-14). If this direct interaction is occurring it appears to be insufficient to cause PS externalization without increased levels of intracellular cAMP. The expansive and extended externalization of PS in primary trophoblasts appears to require the combined effects of forskolin: the stimulation or inhibition of unknown transporters and increased intracellular cAMP. In particular, 1,9-dideoxyforskolin is not capable of stimulating expansive PS externalization without the addition of exogenous cAMP. Expansive PS externalization was

inconsistently observed in the Bewo cell line but the trend towards increased PS efflux with forskolin and the combined treatment of 1,9-dideoxyforskolin plus cAMP was observed. Importantly, Bewo required a Br-cAMP concentration 25 times higher than primary cells for significant fusion to be observed; thus it appears Bewo are far less sensitive to cAMP as a second messenger for fusion than are primary cells. This decreased sensitivity of Bewo cells to cAMP in turn may be related to the inconsistent effects observed on Bewo externalization of PS. Since it has been previously shown in trophoblastic cell lines that PS externalization during forskolin induced differentiation is not associated with increased levels of apoptosis, such externalization appears to be an apoptosis-independent event and forskolin may be useful in answering fundamental questions about what proteins and cellular pathways are involved in PS externalization under non-apoptotic conditions.

Forskolin is used extensively in the trophoblast literature as a differentiation agent of trophoblastic cell lines though, to our knowledge, the pleiotropic effects of forskolin beyond activation of adenylate cyclase have not been widely, if ever, acknowledged. Usage of forskolin as a differentiating agent for Bewo was initially presented by Wice et al. (1990) where only the adenylate cyclase activating properties of forskolin are discussed. We hope that the data presented here will caution investigators against usage of forskolin in

the future without consideration of the multiple direct effects this compound is having on trophoblastic cells.

Though it appears that extensive PS externalization is not involved in trophoblastic cell fusion, our experiments have not directly examined whether transient PS externalization at the site of membrane fusion is occurring in trophoblasts. This pattern of PS externalization has been observed in myoblast and macrophage fusion (8-10). A maximum of 3% of primary CT bound annexin-V over 72 hours of differentiation in contrast to the ~70% of cells that would ultimately become incorporated into multinucleate structures in the same time period when differentiation was stimulated with Br-cAMP. Since the exposure of PS at the actual site of membrane fusion could be very short lived the large disparity in the numbers between cells that will fuse and those that were observed to bind annexin-V does not rule out that this may be occurring. Additionally Adler et al. (1995) have shown in the JAR choriocarcinoma cell line that one monoclonal anti-PS antibody (though not another monoclonal anti-PS antibody) can inhibit forskolin-induced fusion by 40%. Treatment of primary CT stimulated to differentiate with Br-cAMP with a monoclonal anti-PS antibody throughout the 72 h differentiation process failed to inhibit cellular fusion implying that this focused externalization is unlikely. Similarly designed experiments using anti-PS antibodies and annexin-V have been shown to block myoblast formation (9,10). Thus close examination of phosphlipid membrane

localization warrants further investigation in trophoblasts to completely exclude the involvement of externalized PS in the fusion process.

Ultimately the data presented in this chapter suggest that PS externalization is unlikely to be involved in trophoblastic cell fusion and brings to the attention of the reader the pleiotropic effects of forskolin treatment on trophoblastic cells.

3.6 References

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Chapter 4: The Characterization of Fibrocyte-like Cells: A Novel Fibroblastic Cell of the Placenta[3]

4.1 Introduction

The placenta is a highly vascularized organ with an expansive capillary network. This vasculature is important not only for maternal-fetal transport of nutrients, waste and gas, but also for placental growth (1-3). Two essential processes must occur for proper growth of the placenta: (1) the expansion of the syncytiotrophoblastic epithelium (ST) through the differentiation and incorporation of cytotrophoblasts (CT) and (2) the extension of existing placental vasculature via angiogenesis (1,4,5). Importantly, intrauterine growth restriction (IUGR) is associated with both a reduction of the ST surface area and malformation of the villous vasculature highlighting the importance of both these processes for the development of a healthy and productive placenta (6-8).

The microenvironment in which the endothelium is maintained is of utmost importance in angiogenesis. Endothelial cells in the villous placenta interact

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with both the cells of the trophoblastic epithelium and mesenchymal cells of the villous stroma. Thus, contact and cross-talk between placental endothelial cells (PEC) and these two populations of cells determine endothelial cell gene expression, proliferation, apoptosis, and ultimately the extension or regression of the villous vasculature. Therefore, placental vascular homeostasis relies on the response of multiple cell types to alterations in micro-environmental factors.

The villous stroma is comprised largely of fibroblastic cells, both myofibroblasts and fibroblasts in stem villi and immature intermediate villi and fibroblasts alone in mature intermediate villi and terminal villi (9). Additionally placental macrophages, or Hofbauer cells, are a significant component of the villous placental stroma (1-3,10,11). Directly lining the exterior of placental blood vessels are $CD146^{+}$ pericytes, which have been shown to have multi-lineage differentiation potential and hematopoietic and mesenchymal stem cells have also been localized to this area of the villi (12-15).

Previous studies have demonstrated the production of angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), placental growth factor (PlGF), angiopoietin 1 and 2 (Ang-1, Ang-2), and platelet-derived growth factor (PDGF) by villous placental constituents. Many of these angiogenic factors are produced by trophoblasts,

but other cells such as the Hofbauer cell and the fibroblastic cells of the stroma are additional sources (1,2,16-22).

4.2 Objectives

In order to examine potential cross-talk mechanisms between stromal cells and the endothelium we set out to develop methods to isolate stromal cells that were more common to the growing ends of placental villi, thus to sites of expanding endothelium, than to stem villi. Thus we characterized marker expression patterns of stromal cells within the terminal and intermediate villi and identified a population of fibroblastic cells positive for monocyte/macrophage lineage (CD115 and CD14) and fibroblastic markers (TE-7). We then went on to develop an isolation protocol for this cell type. Cells with characteristics similar to these have previously been identified in the adult circulation, and are known as fibrocytes (23). The term fibrocyte was also historically used to distinguish fully differentiated, relatively inactivated fibroblasts but in this thesis the term fibrocyte will refer only to the more modern definition of a fibroblastic cell with monocytic origins and characteristics as carefully defined by Pilling et al. (24,25). Fibrocytes play a role in the angiogenesis of wound healing sites predominantly via paracrine support (23). Thus this study examined the localization, purification, characterization, and angiogenic function of placental fibrocyte-like cells.

4.3 Materials and Methods

4.3.1 Tissue Collection

Term placentas and umbilical cords were collected from the Royal Alexandra Hospital in Edmonton, Canada, after proper ethics approval from the University of Alberta ethics committee and informed consent was obtained. Placental samples were obtained from cesarean sections of uncomplicated pregnancies at 39 weeks gestation and were delivered to the laboratory within 30 minutes of delivery for cell isolation. A minimum of eight randomly selected pieces were fixed in either 4% pfa or periodate-lysine-paraformaldehyde (PLP) for 1 h and then immersed in 30% sucrose (Sigma, Oakville, ON) for 24 hours. The tissue was then frozen in OCT (Sakura Tissue-Tekat, Torrance, CA) at -70^{o}C and 5 μm sections were cut on a Shandon tissue cryostat (Thermo Fisher Scientific, Pittsburg, PA).

4.3.2 Cellular Isolation of Fibrocyte-like cells

Placental fibrocyte-like cells were isolated during our efforts to separate villous fibroblastic cells from Hofbauer cells. This led to the development of isolation methods for fibrocyte-like cells from term tissue by two methods: (1) adherence selection of unsorted villous components and (2) isolation of fibrocyte-like cells by CD45-positive cell sorting.

4.3.2.1 Adherence selection of unsorted villous components

All samples were isolated by serial trypsin-DNase digestion of minced placental tissue followed by a red blood cell lysis as detailed in (26). Cells were then plated in IMDM supplemented with 10% FCS and antibiotics (IMDM-FCS) in a 100mm culture dish for 1 h in a fully humidified atmosphere with 5% CO_2. Non-adherent cells were then washed away with PBS, the medium replaced and the cells incubated overnight. Medium was replaced every 2-3 days and after 10-14 days in culture cells morphologically resembling fibroblasts (long, thin, spindle shaped cells) became the predominant type of cell with the balance of the cells in the culture at this time displaying a myofibroblastic phenotye (large, kite shaped cells) or characteristic macrophage phenotype of Hofbauer cells. Cells were passaged with 0.05% trypsin-EDTA (Gibco) and by passage 2 the cultures were >70% cells with spindle shaped fibroblastic cellular morphology.

4.3.2.2 Isolation by CD45 positive cell sorting

We went on to develop a method for isolation of a purer population of cells with the same morphological features (long, thin, spindle shaped cells). After the serial tissue trypsin digestion and removal of non-adherent cells from the 100mm culture dishes, the cells were incubated overnight and then released from the culture dish with 0.05% Trypsin-EDTA (Gibco) and prepared for FACS sorting. Briefly, non-specific binding was blocked with a 15min incubation in 20% FCS in PBS on ice followed by labeling of the cells with mouse anti-human CD45-FITC (Biolegend, San Diego, CA, USA; 1:20), for

20min on ice in 10% FCS in PBS in the dark. Cells were then separated on a FACSAria flow cytometer (BD, Mississauga, ON) and CD45-labeled cells were collected. Sorted cells were plated in IMDM-FCS and incubated in a fully humidified atmosphere at 20% O_2 5% CO_2. After 10-14 days a population of confluent spindle shaped highly proliferative cells emerged and were passaged. After this passage the culture completely lacked cells with a monocyte/macrophage morphology and all cells were of a spindle shaped fibroblastic morphology.

4.3.3 Cellular Isolation of Villous Placental Endothelial Cells (PEC)

Placental endothelial cells (PEC) were isolated from term placentas by the method of Leach et al. (1994) (27) modified as follows. Villous trees (20mL of packed material) were isolated, rinsed in Hank's medium (HBSS, Gibco) and re-suspended equally into two 25mL aliquots of pre-warmed trypsin (0.12%, Gibco) in HBSS. After a 4 min incubation at 37^{o}C with frequent shaking, the supernatant containing trophoblasts was discarded. The trees were then macerated finely and incubated in pre-warmed trypsin (0.12%), pronase (0.06%, Sigma, St. Louis, MO) and DNAse (5U/mL, Sigma) for 10 min at 37^{o}C with frequent shaking. The suspension was filtered through a 40 mesh sterile screen and then through several folds of sterile gauze. The filtrate was centrifuged to retain the pellet, the cells re-suspended in 3mL of 20% goat serum (Jackson Labs, Bar Harbour, MA) in HBSS, incubated on ice for 15 min, re-suspended in 20mL 2% FCS in HBSS and then slowly centrifuged (5 min, 1000 rpm on a Beckman GS-6R tabletop centrifuge, Beckman Instruments,

Palo Alto, CA). The pellet was re-suspended in 1 mL of 5% FCS in HBSS, $4x10^6$ CD31 Dynalbeads (Invitrogen) added, and the cells and beads agitated by continuous rocking on ice for 25min followed by magnetic separation. The supernatant containing non-beaded cells was aspirated, the cells re-suspended in 1.5mL of 0.1% BSA in 5% FCS/HBSS, magnetically separated and supernatant again discarded. This washing step was repeated 7 times. The washed cells were transferred with 2.5mL 5% FCS in EBM-2MV medium with complete supplements (Lonza, Walkersville, MD) to a T25 flask (Corning, Corning, NY) pretreated with 0.2% gelatin (Sigma) and incubated in an incubator at 6% O_2, 5% CO_2. After 24 h medium was removed and cells were gently washed to remove non-adherent cells and unbound beads and medium was replaced. Medium was replaced every 2-3 days and when cells were nearly confluent they were released with 0.05% trypsin-EDTA and again subjected to magnetic bead separation as described above. The cells were then grown to confluence in gelatin-coated T25 flasks and harvested for Matrigel assays or cryopreservation. At all times the medium was pre-equilibrated to the proper oxygen tension by pre-incubation for a minimum of 3 h before addition to the cells. The endothelial nature of these cells was established through positive staining for Von Willebrand's factor, endoglin (CD105), CD31, binding of UEA lectin, and the uptake of dil-Ac-LDL (data not shown).

4.3.4 Immunofluorescence

The expression of fibroblast and monocyte/macrophage markers in villous tissue sections and isolated spindle shaped fibrocyte-like cells was shown using immunofluorescence. Isolated fibrocyte-like cells were fixed with either 4% pfa or PLP for 10 min followed by a washing step with PBS. Isolated cells or tissue sections were then incubated for 1 h with 20% NGS in PBS. Antibodies against CD45, CD115, CD14, PM-2K, TE-7, CD31, Collagen IV, and VEGFR-2 (see table 4-1 for concentrations and suppliers) or *Ulex europaeus* lectin (UEA lectin; 20µg/mL; Sigma) and their appropriate isotype controls were incubated overnight at 4^{o}C. After washing with PBS Alexafluor 488 or 546 goat anti-mouse or rat (Invitrogen), or Alexafluor 546 Strepavidin were added at 1:200 dilution and incubated for 1 h at room temperature. Nuclei were visualized with DAPI as outlined in section 2.3.4.

Table 4-1: Antibodies used for FACS and immunofluorescence

Antigen	Purpose	Ig Type/Source	Dilution/Conc.
CD45	FACS	Mouse IgG_1, Biolegend	1:20
CD45	IF	Mouse IgG_{2a}, Gift from Dr. Hanne Ostegaard	1:3000/ 1 μg/mL
Collagen IV	IF	Mouse IgG_1, Sigma	1:500
CD31	IF	Mouse IgG_1, Dako	1:30
CD115	IF	Mouse IgG_1, Santa Cruz	1:100/ 2 μg/mL
CD115	IF	Rat IgG_1, Thermo Scientific	1:100/ 10 μg/mL
CD14	IF	Mouse IgG_1, In house clone 82H3	1:100
VEGF-R2	IF	Mouse IgG_1, R&D Systems	1:20; 20 μg/mL
PM-2K	IF	Mouse IgG_1, AbD Serotec	1:200
TE-7	IF	Mouse IgG_1, Chemicon	1:200
FITC conjugated Isotype control	FACS	Mouse IgG_1, Santa Cruz Biotechnology	1:40
Isotype control	IF	Mouse IgG_1, R&D Systems	2-20 μg/mL
Isotype control	IF	Mouse IgG_{2a}, R&D Systems	1 μg/mL
Isotype control	IF	Rat IgG_1, Santa Cruz Biotechnology	10 μg/mL
Alexa Fluor Goat anti-mouse 488	IF	Goat IgG, Invitrogen	1:100-1:200
Alexa Fluor Goat anti-rat 488	IF	Goat IgG, Invitrogen	1:200
Alexa Fluor Goat anti-mouse IgG_1 546	IF	Goat IgG, Invitrogen	1:100-1:200

IF=immunofluorescence;

For double-immunofluorescent labeling with two mouse antibodies tissue sections were blocked with 20% NGS for 30 min at room temperature followed by a 90 min incubation with one mouse antibody and the appropriate isotype control at room temperature. After washing with PBS a 1:100 dilution of Alexafluor 488 goat-anti mouse was added for 30 min and the cells or slides were again washed with PBS. There was then a second 30 min blocking step with 20% NGS followed by another 90 min incubation with the second mouse antibody (IgG_1 isotype only) and isotype control. Slides were then washed again with PBS and incubated with a 1:100 dilution of Alexafluor 546 goat-anti mouse IgG_1 for 30 min. Nuclei were then visualized with DAPI as in 2.3.4. Fluorescent images were obtained with an Olympus

IX81 motorized inverted research microscope equipped with a Lambda DG-4 high-speed filter changer and a Cascade 16 bit digital monochrome camera (Olympus, Melville, NY) using a 10x objective. Slidebook 3.0 software (Carsen, Markham, ON) was used to capture and export images. Brightfield images of unstained fibrocyte-like cells were acquired on a Zeiss Axiovert 200M fluorescent microscope using a 10x objective lens and Axiovision 4.5 software (Zeiss, Toronto, ON).

4.3.5 Phagocytosis Assay

The ability of fibrocyte-like cells to phagocytose was tested with latex beads by incubating 80% confluent cells with FITC-conjugated latex beads (0.51μM diameter, Polysciences, Warrington PA) diluted in IMDM-FCS. Cells were incubated for 1h at 37°C at 20 or 6% O_2, 5% CO_2 followed by at least 6 vigorous washes with PBS. Cells were visualized using a Zeiss Axiovert 200M fluorescent microscope using a 10x lens and images were captured using both brightfield and FITC excitation in AxioVision 4.5.

4.3.6 Collection of Conditioned Medium

Medium was removed from 80% confluent fibrocyte-like cells and they were rinsed twice with PBS. Medium was replaced with serum-free EBM-2 basal medium and the cells were incubated overnight at 8% O_2 5% CO_2. The fibrocyte-like cell-conditioned medium (FcCM) was then collected, centrifuged at 550g in a Beckman GS-6R centrifuge, filtered through a Millex-HV PVDF filter (0.45 μM; Millipore, Bedford MA), and frozen at -70°C until

use. Cell-free conditioned medium was collected from cells up to passage 10. When cells had been moved from one oxygen tension to another (20% to 8%) conditioned medium was not collected until the cells had been maintained at the new oxygen tension for a complete passage.

4.3.7 Angiogenesis Assay

Growth factor reduced Matrigel (GFR-Matrigel; BD, Bedford, MA) was thawed overnight on ice at 4^{o}C and 40μL of GFR-Matrigel was added to each well of a 96 well plate (Nunc, Roskilde, Denmark) followed by a 30 min incubation at 37^{o}C. Meanwhile PEC were released with 0.05% Trypsin-EDTA, centrifuged and re-suspended in either EGM-2MV [containing manufacturer recommended concentrations of all supplements including: FBS, b-FGF, VEGF, Insulin-like Growth Factor (IGF), epidermal growth factor (EGF)], serum free basal EBM-2 medium, or FcCM. The cells were then plated onto the solidified GFR-Matrigel at a density of $10x10^3$ cells per well and incubated at an atmosphere of 8% O_2 5% CO_2 for 6 h. Endothelial tubules were fixed with 4% pfa for 10min and three brightfield images were obtained per well using a 10x objective lens on a Zeiss Axiovert 200M fluorescent microscope. Duplicate wells of each treatment were prepared and experiments were carried out on endothelial cells from at least two separate samples and with conditioned medium from fibrocytes isolated from greater than three different placentas. Total area of each image covered by tubule-like structures was measured by outlining the tubule-like structures while excluding cellular islands with AxioVision 4.5 software and the average of the

three images obtained per well was calculated to represent the area covered by tubule-like structures.

4.3.8 Blocking Antibodies

Neutralizing antibodies directed against VEGF and b-FGF (Goat IgG, R&D Systems; Minneapolis, MN) were used to identify the presence of individual growth factors and their involvement in PEC tubule formation. Fibrocyte-like cell conditioned medium was thawed and neutralizing antibody or goat IgG was added to the conditioned medium (final concentrations: anti-VEGF 5μg/mL; anti-b-FGF 1μg/mL; Goat Ig 10μg/mL). All media was pre-incubated for 1 h at 37^{o}C 8% O_2 5% CO_2 before addition to the cells. Trypsinized PEC were then re-suspended directly into either control or test medium and the same protocol used for angiogenesis assays was carried out.

4.3.9 ELISA Analysis

Detection of angiogenic growth factors in fibrocyte-like cell conditioned medium was carried out using a multiplex ELISA for b-FGF, PlGF, VEGF, and s-Flt (Meso Scale Discovery, Gaithersburg, MD). Growth factor concentration was normalized for each sample to the total amount of protein in the cell lysate the FcCM was produced in. Protein concentration in was determined by a Bradford protein assay (Biorad, Hercules, CA).

4.3.10 Statistical Analysis

Multiple un-paired t-tests were used to analyze the effect of FcCM on tubule-like structure formation with a $p<0.01$ being considered significant. A one-

way ANOVA was used to compare groups of means with a Bonferroni post-hoc to compare the groups in the neutralizing antibody assay with a $p<0.05$ being considered significant. Statistics were performed using Prism 4.0 (Graphpad Software, San Diego CA).

4.4 Results

4.4.1 In vivo marker expression patterns in the fibroblastic stroma

Sections of human term placenta were stained with a panel of antibodies directed against the monocyte/macrophage markers CD115, CD14 and the mature macrophage marker PM-2K, the hematopoietic cell marker CD45 and the fibroblast marker TE-7 as well as for binding of UEA lectin. This staining showed a large pool of spindle-shaped cells within the villous stroma positive for CD115, CD14, and TE-7 (Figure 4-1). Cells expressing this combination of markers were found throughout the stroma of the terminal villi and, importantly, at an appreciably lower density in the stem villi (data not shown). Spindle-shaped $CD115^+$ $CD14^+$ $TE\text{-}7^+$ cells were not found to express CD45 or the mature macrophage marker PM-2K. PM-2K expression was limited to $CD45^+$ cells (Figure 4-1L) and CD45 expression was never seen to co-localize with the fibroblast marker TE-7 (Figure 4-1I).

4.4.2 Marker expression and phenotypic characteristics of isolated cells

A high proportion of cells isolated through adherence selection of trypsin-digested villous components were of spindle-shaped fibroblastic morphology (>70%) and the cells isolated by both adherence selection and CD45 FACS

sorting were positive for the monocyte/macrophage markers CD115 and CD14 as well as the fibroblast marker TE-7 (Figure 4-2). To further purify this previously unidentified cell type, cells from term placentas were isolated by FACS for CD45 expression. The FACS sorted population contained predominantly cells with a macrophage morphology directly after sorting but gradually changed during the 10-14 day culture to a population that was $CD115^+$, $CD14^+$, and $TE\text{-}7^+$ (>95%), spindle-shaped but CD45 negative (Figure 4-2). The CD115+, CD14+, TE-7+ cells were highly proliferative after 10-14 days of culture with an approximate doubling time of 48 h. Most cells were also weakly positive for VEGF-R2 (KDR) (>95%), negative for CD31 (a macrophage and endothelial marker) and negative for the mature macrophage marker PM-2K (Figure 4-2). The cells were negative for the pericyte marker CD146 and had variable expression of smooth-muscle actin (data not shown). Additionally the CD115+, CD14+, TE-7+ cells produced collagen IV and phagocytosed latex beads (Figure 4-2). In adherence selected cells we also observed myofibroblastic kite-shaped cells that were unable to phagocytose the latex beads and thus easily distinguishable (Figure 4-2J). Thus, we isolated a spindle-shaped population of cells that morphologically and functionally resemble fibroblasts but express monocyte/macrophage markers, can carry out phagocytosis, and are derived from CD45 positive (hematopoietic) cells.

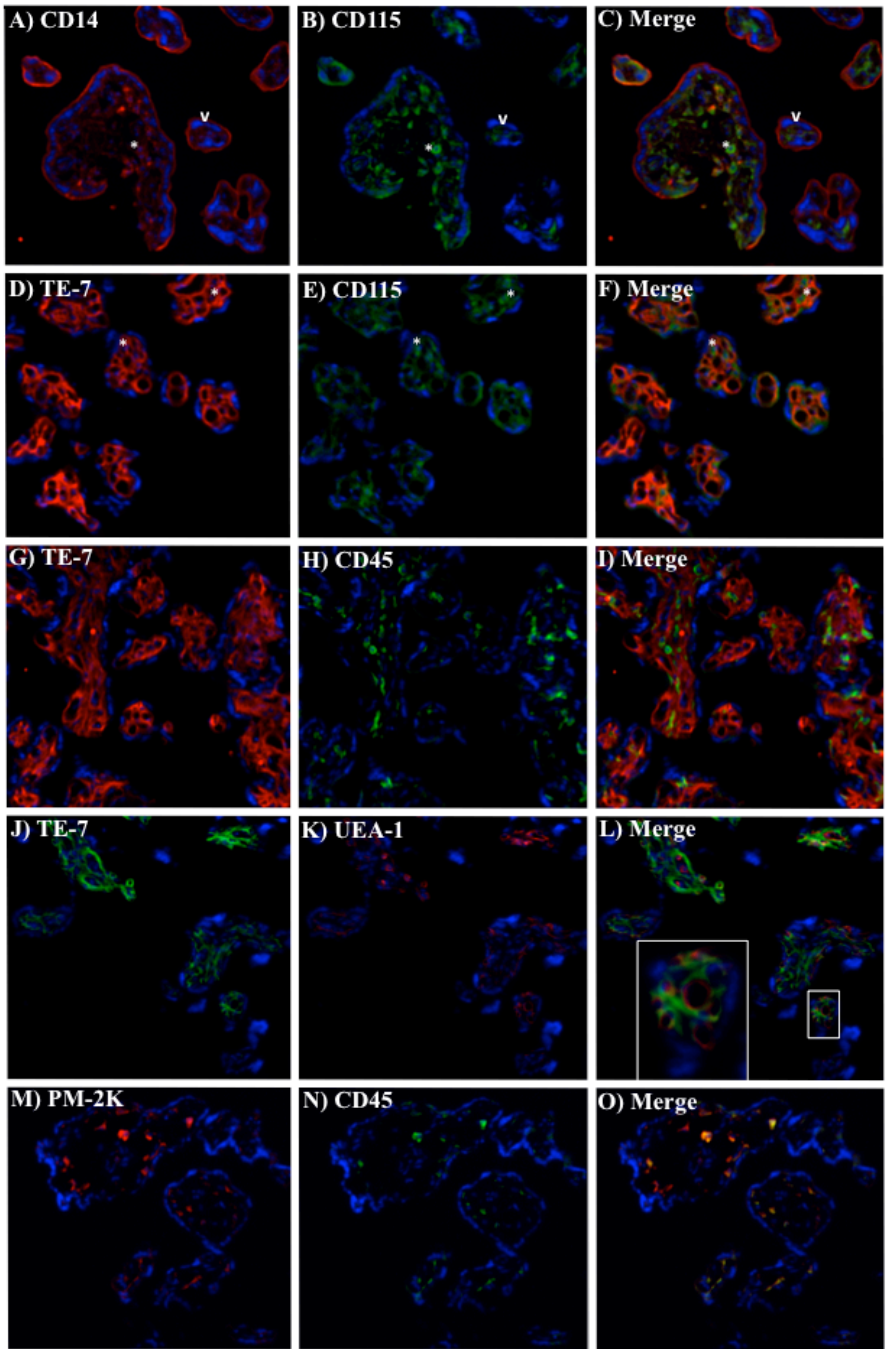

Figure 4-1: Immunofluorescent identification of fibrocyte-like cells in term placenta

A) Monocyte/macrophage marker CD14; B) Monocyte/macrophage marker CD115 (CSF-1R); C) Merged image; * identifies Hofbauer cells; v identifies syncytiotrophoblast; D) Fibroblast marker TE-7; E) CD115; F) Merged image; * identifies Hofbauer cells; G) TE-7; H) Hematopoietic cell marker CD45; I) Merged image; J) TE-7; K) Binding of UEA lectin; L) Merged image with inset high magnification image of a terminal villi; M) Mature macrophage marker PM-2K; N) CD45; O) Merged image. These images are representative of images taken from > 5 placentas.

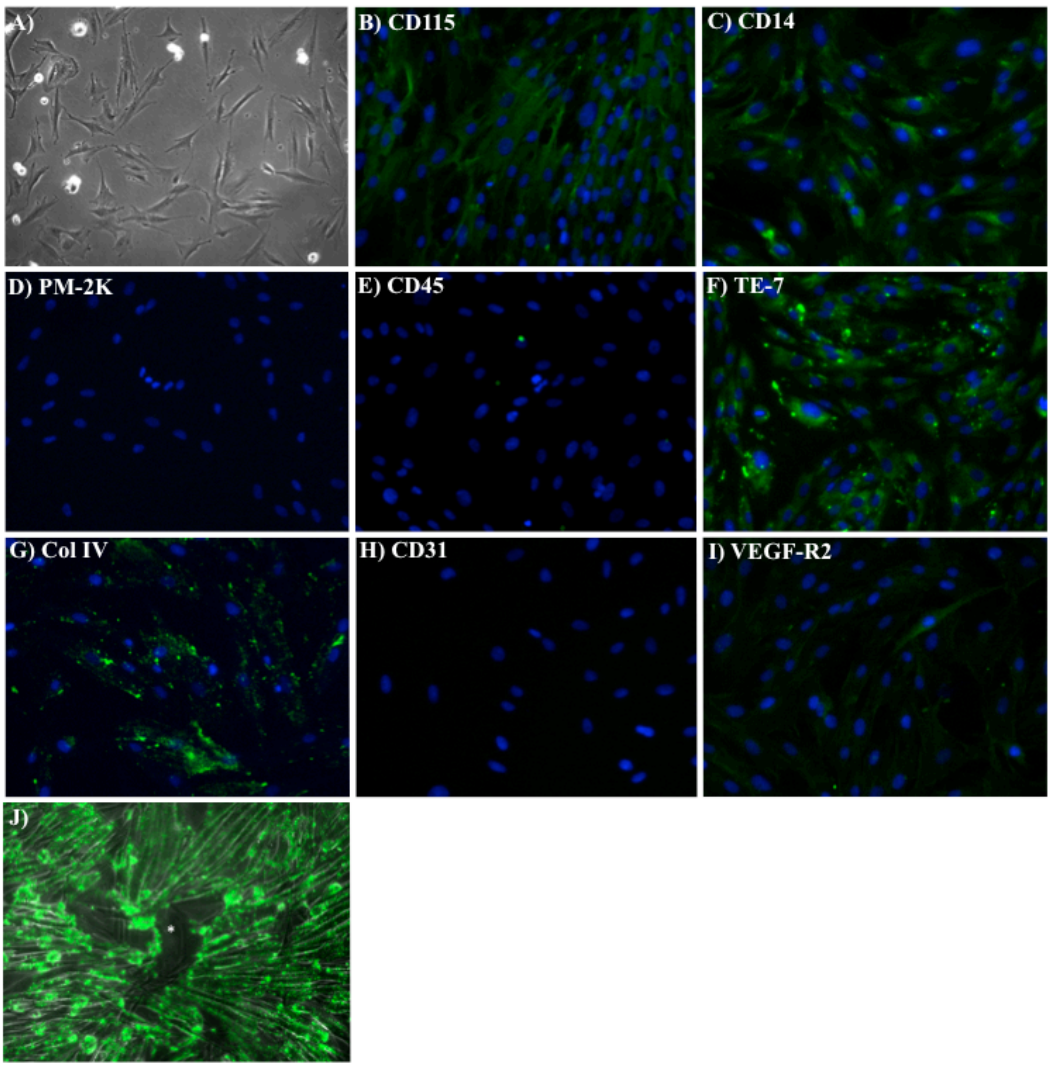

Figure 4-2: Immunofluorescent characterization of markers expressed by fibrocyte-like cells isolated from human term placenta *in vitro*
A) Brightfield image of cellular morphology; B) Monocyte/macrophage marker CD115 (CSF-1R); C) Monocyte/macrophage marker CD14; D) Mature macrophage marker PM-2K; E) Hematopoietic cell marker CD45; F) Fibroblast marker TE-7; G) Collagen IV; H) Endothelial cell marker CD31; I) VEGF-R2 (KDR); J) Phagocytosis of isolated fibrocytes of fluorescently labeled latex beads; * indicates a non-phagocytic kite shaped myofibroblastic cell. These images are representative of triplicate images taken from cells isolated from >10 placentas.

4.4.3 The angiogenic potential of isolated fibrocyte-like cells

Due to the localization of $CD115^+$ $CD14^+$ $TE\text{-}7^+$cells adjacent to the villous vasculature, the capacity of adult fibrocytes to support angiogenesis and previous publications showing the production of angiogenic growth factors

in the placenta by cells with the same localization as fibrocyte-like cells, we assayed the ability of fibrocyte-like cell conditioned medium (FcCM) to support endothelial cell tubule formation. FcCM increased the formation of PEC tubule-like structures on Matrigel by 2-fold compared to untreated medium (Figure 4-3).

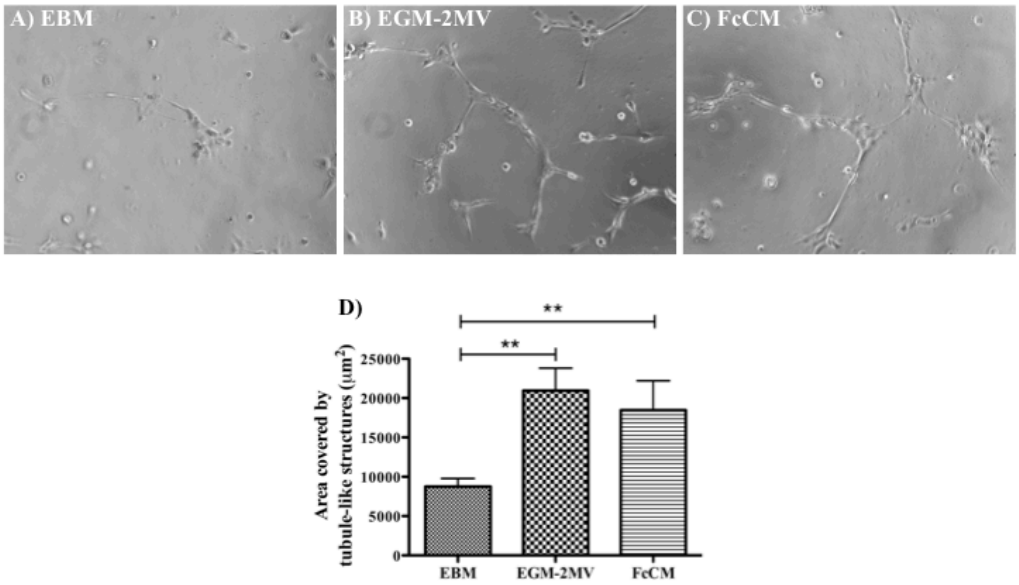

Figure 4-3: Placental endothelial cell tubule-like structure formation is induced by fibrocyte-like cell conditioned medium on growth factor-reduced Matrigel after 6h
A) EBM basal endothelial cell medium; B) EGM-2MV endothelial cell medium containing growth factors; C) FcCM placental fibrocyte-like cell conditioned medium; D) Summary graph of tubule-like structure area; Bars= Mean ± SEM (n=4; unpaired t-test **= vs. EBM $p < 0.01$).

This observation suggested that FcCM contained factors that regulated PEC tubule formation. FcCM was assayed by ELISA for possible regulators of angiogenesis and the pro-angiogenic factors VEGF (median 49.55 pg/mg; range 15.28-71.76 pg/mg) and b-FGF (median 0.92 pg/mg; range 0.31-2.47 pg/mg) but no PlGF (below detection limit of assay; <10 pg/mL) and the anti-angiogenic factor sFlt (median 6.98 pg/mg; range 0-31.88) were identified.

We assessed the contribution of VEGF and bFGF to positively stimulating tubule-like structure formation with neutralizing antibodies and found that individually eliminating either factor reduced tube formation to control levels (Figure 4-4).

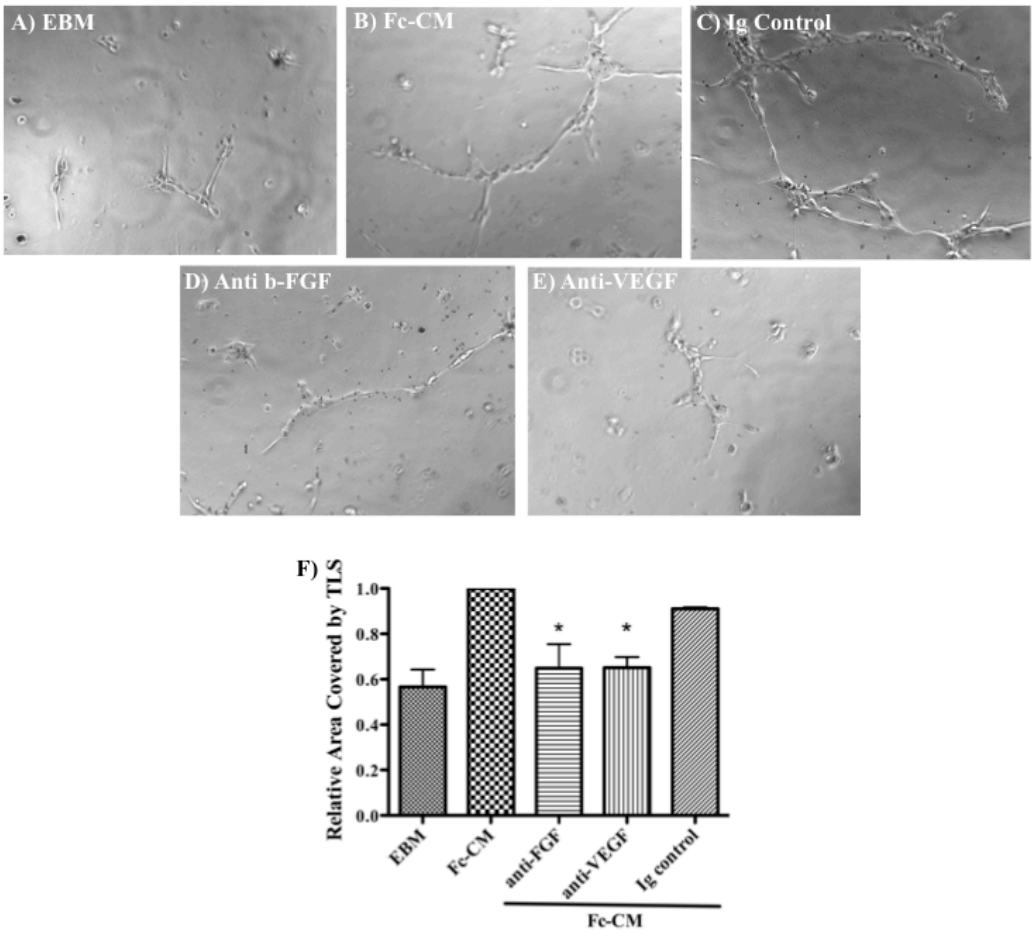

Figure 4-4: Placental endothelial cell tubule-like structure formation induced by fibrocyte-like cell conditioned medium can be reduced by the addition of neutralizing antibodies to VEGF and b-FGF but not by isotype matched control
A) EBM basal endothelial cell medium; B) Fc-CM placental fibrocyte-like cell conditioned medium; C) FcCM plus Goat IgG isotype control; D) FcCM plus anti- b-FGF; E) FcCM plus anti-VEGF; F) Summary graph of relative tubule-like structure area; Bars= Means ± SEM (n=4; ANOVA *= vs. FcCM $p<0.05$).

4.5 Discussion

In this study we identified the presence of fibrocyte-like cells in the villous placenta and the capacity of placental fibrocyte-like cell conditioned medium to induce endothelial cell tubule formation. Fibrocytes are hematopoietic lineage-derived spindle shaped fibroblast-like cells that are recruited from the circulation to sites of tissue injury early in wound healing where they proliferate and contribute to tissue repair and fibrosis (25,28,29). Fibrocytes derived from adult peripheral blood have been shown to produce b-FGF, VEGF and PDGF-aa and the conditioned medium from fibrocytes induces endothelial cell proliferation, migration and tubule formation on Matrigel as well as stimulating blood vessel formation in Matrigel plugs implanted in mice (23,30). In this study the stimulation of tubule-like structure formation by fibrocyte-like cell conditioned medium is reduced nearly to base line levels with neutralizing antibodies against VEGF and b-FGF and contains both angiogenic and anti-angiogenic growth factors. Cells staining for macrophage markers but morphologically resembling fibroblasts within the placenta have been noted in the literature but no further characterization of these cells has been undertaken (10,11). Our observations and the literature indicate that fibrocyte-like cells are an important component of the villous placental stroma and are likely to be involved in the paracrine regulation of placental angiogenesis.

Due to the absence of a single marker to distinguish fibrocytes, the currently accepted definition for the identification of these cells in adults relies on the expression of a combination of hematopoietic and stromal cell markers. The most common markers used in the literature are the hematopoietic marker CD45 and the stem cell marker CD34 or chemokine receptor CXCR4 in combination with the production of collagen I (25,28,29,31). Pilling et al. (25) carefully identified markers that can distinguish between monocytes, macrophages, fibrocytes, and fibroblasts, and by their definition fibrocytes should be $CD45^{+}$, 25F9 (macrophage marker), and S100A8/A9 (macrophage marker) positive, but negative for the macrophage marker PM-2K. They identify PM-2K as a marker only found on macrophages and importantly also identified TE-7 as a fibroblast specific marker (25). Based on this information, in this communication we used a combination of the expression of monocyte/macrophage markers CD14, CD115 and PM-2K, the hematopoietic lineage marker CD45, and the fibroblast marker TE-7 to identify fibrocyte-like cells in cross sections of term placenta. Three of the markers CD115, CD14 and TE-7 have overlapping expression in a large population of cells within the placental stroma. Monocyte/macrophage markers identify two separate populations within the villous stroma: Hofbauer cells which are CD45, CD14, CD115, and PM-2K positive and the stellate string-like population of morphologically fibroblastic cells that we identify as fibrocyte-like cells. We found the expression pattern of CD14, a classical monocyte/macrophage marker, to be widespread in the placenta

with much of the villous stroma labeling via immunofluorescence. This has previously been reported by Sutton et al. and we have previously reported the expression of CD14 by the villous syncytiotrophoblast (11,32). Although this is not the characteristic pattern of expression in adult tissues, CD14 can also be found in a soluble form; thus, this may be an explanation for the wide distribution throughout the stroma (33). Due to the widespread expression of CD14 we utilized a second monocyte/macrophage marker, CD115, the receptor for macrophage colony stimulating factor (M-CSF/CSF-1). Interaction between CSF-1 and CD115 is a potent inducer of monocyte/macrophage lineage differentiation and proliferation and thus CD115 has a functional role in the maintenance of macrophage lineage cells (34). The mature macrophage marker PM-2K was selected to distinguish potential fibrocytes from Hofbauer cells as per Pilling et al. (25). CD68 was not used as a macrophage marker because fibroblasts have been show to variably express it (25). Due to the lack of expression of CD45 and the expression of the fibroblastic marker TE-7 we cannot call the cells we have identified fibrocytes by the Pilling definition and thus have designated them fibrocyte-like. Fibrocytes (expressing CD45 and fulfilling the general definition described above) have previously been identified in the human umbilical cord and chorionic vessels (35). The fibrocyte-like cells identified here share the key characteristic of being derived from the hematopoietic lineage with these cells, but express markers (such as TE-7) that fibrocytes do not, and thus represent a novel population.

The localization of fibrocyte-like cells within the intermediate and terminal villi of the term placental stroma overlaps with the localization of stromal cells previously designated as reticulum cells. Reticulum cells were identified to differ from the fibroblastic cells that form the stroma of stem villi and have been shown to line the stromal channels of the intermediate villi and lie between the capillaries of the terminal villi (36,37). The immunofluorescent panel of markers utilized in this study stain a specific population of cells with the same localization, thus we propose that the cells designated as fibrocyte-like cells may be reticulum cells.

The markers used to identify fibrocyte-like cells *in situ* were expanded in the examination of cultured cells. It was found that the isolated population of fibroblastic cells consistently expressed $CD14^+$ $CD115^+$ $TE\text{-}7^+$ but were PM-2K and CD45 negative were capable of phagocytosis of plastic beads and the production of collagen IV. Collagen IV production was selected instead of collagen I production because collagen I is absent in some areas of the placenta and collagen IV is strongly expressed throughout the stroma (38). Cultured fibrocyte-like cells were also consistently negative for CD31 (PECAM-1), an endothelial cell marker that is also expressed on macrophages, and were weakly positive for VEGFR-2 (25). The derivation of these cells from cultures of highly purified CD45 FACS sorted cells indicates that they originate from hematopoietic precursors despite their ultimate lack of expression of the hematopoietic marker. Progressive loss of CD45

expression in cultured fibrocytes has been noted in the literature and the marker expression patterns observed in our cultured cell population align exactly with the populations of cells identified in our *in vivo* data (25,31). Recently, Hofbauer cells in mixed mesenchymal culture were observed to rapidly change from a macrophage phenotype to a fibroblast phenotype in vitro (39). Additionally, Ingman et al. note that some fibroblastic cells within mixed mesenchymal cultures are capable of phagocytosis (39). These independent observations support two of our findings and suggest that fibrocyte-like cells may differentiate directly from Hofbauer cells in the placenta or from circulating fetal fibrocytes.

In conclusion, we have identified the presence of fibrocyte-like cells within the human villous placenta and the ability of fibrocyte-like cell conditioned medium to support endothelial cell tubule-like structure formation *in vitro*. This previously uncharacterized cell type may play an important role in placental angiogenesis and in the overall growth of the placenta.

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Chapter 5: Fibrocyte-like cells from intrauterine growth restriction placentas have a reduced ability to stimulate angiogenesis[4]

5.1 Introduction

It is generally accepted that fetal and placental weights are directly related. This relationship has primarily been established due to experimental reduction of placental size in sheep, but can be clearly evidenced in the common human pregnancy complication of intrauterine growth restriction (IUGR) (1,2). In IUGR, a fetus fails to achieve its genetic growth potential for a given gestational age and is therefore pathologically small (3). The placental weight of infants born IUGR has been shown to be reduced by 24% compared to infants with appropriate for gestational age (AGA) growth and the actual placental size of IUGR infants has been shown to be reduced compared to AGA infants of the same birth-weight (1).

Two essential processes control the overall growth of the placenta and thus the growth of the fetus: 1) the expansion of the trophoblastic epithelium via a

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- Riddell, M.R., Winkler-Lowen B., Jiang Y., Guilbert, L.J., and S.T. Davidge. Fibrocyte-like cells from intrauterine growth restriction placentas have a reduced ability to stimulate angiogenesis. Accepted for publication in American Journal of Pathology.

Contribution: Riddell M.R. was the project coordinator, performed all the experiments and data analyses, wrote the first draft of the manuscript (including all the figures) and coordinated with the other authors in compiling the final version of the manuscript.

process of differentiation and 2) the extension of the placental vasculature by angiogenesis. IUGR is associated with alterations in both trophoblast differentiation and placental angiogenesis (4-8). Profound alterations in the structure of the terminal villous vasculature have been observed in IUGR placentas. Krebs et al. (1996) documented a reduction in vascular branching and elongation of the terminal villous vasculature specifically in cases of IUGR with absent or reversed end diastolic (ARED) umbilical artery waveforms in perfusion fixed tissue by electron microscopy (9). Peripheral villous (intermediate and terminal villi) capillary volumes, surface area, and lengths have also been found to be decreased in multiple sterological studies (10-13). Both an increase (in cases of IUGR with persistent end diastolic umbilical artery flow) and decrease (in cases of IUGR with ARED) in peripheral villous capillary branching have been observed utilizing stereology (14). Thus IUGR is associated with significant alterations in angiogenesis manifesting as structural alterations in the vasculature, but the mechanisms through which these alterations occur remain to be elucidated.

One of the predominant hypotheses on the etiology of IUGR is that it is caused by insufficient trophoblastic remodeling of the maternal spiral arteries during the first and early-second trimester which in turn reduces maternal blood supply to the developing feto-placental unit. This reduction in oxygen delivery to the placenta is hypothesized to result in the morphologic alterations observed in the established disease. Paradoxically hypoxia is a

well-established inducer of angiogenesis. Hypoxia stimulates the induction of important pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), through the hypoxia-inducible factor (HIF) pathway (15). This ultimately stimulates angiogenesis to occur in order to increase the oxygen supply to an area. Once sufficient angiogenesis has occurred and tissue hypoxia has abated the vasculature returns to its normally quiescent state. Angiogenesis is clearly deficient in IUGR placentas, but since placental tissue cannot be collected until IUGR is already established the effects of hypoxia on placental angiogenesis over time is unknown. Whether hypoxia may initially stimulate placental angiogenesis early on in the progression of IUGR, but has the opposite effect after chronic long-term exposure resulting in the reduced angiogenesis observed by delivery, or whether the placentas of fetuses that develop IUGR do not respond in similar ways as those from a fetus which achieves normal growth are important questions to address. We hypothesize that chronic long-term unresolved hypoxia would ultimately result in cessation of angiogenesis due to a focus on cell survival in the sub-optimal environment instead of sustaining the classical pro-angiogenic response for prolonged periods. This is most likely to manifest as a change in the balance of pro- and anti-angiogenic factors after chronic hypoxic exposure (days to weeks) and could manifest differently in the different cellular constituents of the placenta and result in the overall decreased angiogenesis that has been repeatedly observed in IUGR placentas.

The cell types that are involved in stimulating placental angiogenesis are also largely unknown. The endothelium itself has a key role in integrating and responding to micro-environmental signals but other cellular components within the placenta likely play important roles through physical interaction with the endothelium and through the production of secreted pro and anti-angiogenic factors. The predominant site of angiogenesis within the placenta is at the level of the terminal villi (16). This villous type is dominated by the vasculature, with greater than 50% of the villous volume taken up by blood vessels (17,18). The endothelium is therefore in very close proximity to the epithelial trophoblast layer, which undoubtedly has a role in controlling angiogenesis, though the mechanisms remain to be defined. As presented in Chapter 4, we have identified the predominant type of fibroblastic stromal constituent within the terminal villi as fibrocyte-like cells. It was also shown in Chapter 4 that isolated fibrocyte-like cell conditioned medium (FcCM) is capable of stimulating PEC angiogenesis *in vitro.* Thus this cell type is also very likely to play an important role in placental angiogenesis.

5.2 Objectives

We were therefore interested to understand whether fibrocyte-like cells from IUGR placentas have the same capacity to stimulate angiogenesis as those from a normal placenta and whether maintenance in chronic hypoxia would effect the ability of both normal and IUGR fibroctye-like cells to stimulate angiogenesis. We hypothesized that FcCM from IUGR placentas would have decreased ability to stimulate angiogenesis *in vitro* and that maintenance of both normal and IUGR fibroctye-like cells in chronic hypoxia would result in a decrease in their ability to stimulate angiogenesis.

5.3 Materials and Methods

5.3.1 Tissue Collection

All placentas were collected from the Royal Alexandra Hospital in Edmonton, AB Canada after proper ethical approval from the University of Alberta ethics committee and informed consent was obtained. Normal control samples were collected from singleton uncomplicated term pregnancies. IUGR samples were collected from pregnancies where the fetus had a decrease in growth trajectory documented by serial ultrasound assessment either in the presence or absence of an asymmetric growth pattern. IUGR samples were also confirmed TORCH negative and to have a predicted fetal weight by ultrasound that fell below the tenth percentile. All placentas were transported to the laboratory and isolation of cells was started within one hour of birth. Normal term placental samples were used as a control population in order to eliminate the potentially confounding effects of other causes of prematurity.

5.3.2 Fibrocyte-like Cell Isolation

Fibrocyte-like cells were isolated as presented in section 4.3.2.

5.3.3 Placental Endothelial Cell Isolation

PEC were isolated from normal term placentas as previously described in section 4.3.3. PEC cultures were then maintained at 6% O_2 5% CO_2. This

oxygen content was found to be essential for PEC growth for isolation was not successful when the cells were maintained at higher oxygen concentrations (8-21% O_2).

5.3.4 Production of Fibrocyte-like cell Conditioned Medium (FcCM)

Fibrocyte-like cells were maintained at either a fully humidified atmosphere of 6% O_2 5% CO_2 (normoxia) or 1% O_2 5% CO_2 (hypoxia) in a Forma model 3130 incubator (Marrieta, OH) at 37^{o}C and were maintained for a complete passage under these atmospheric conditions before conditioned medium was collected (~ 1 week). Conditioned medium was collected as presented in section 4.3.7. Fibrocyte-like cells were used to make FcCM between passages 2-8.

5.3.5 Endothelial Cell Migration Assay

PEC were trypsinized and 80,000 cells were seeded in EBM-2+0.2% BSA (supplement free basal endothelial cell medium) in the top of a human fibronectin coated transwell insert (12 well plate; 3µm pore size; BD; Bedford MA). FcCM or EBM-2 was made 2% with FCS, antibiotics were added, and then the medium was placed in the bottom of each well alongside an EGM-2MV positive control. The plate was incubated at 6% O_2 5% CO_2 for 24 hours. Inserts were then fixed in methanol, cells remaining on the top of the insert were removed with cotton swabs and inserts were then stained with haematoxylin. The total number of cells that had migrated to the bottom side of the insert were manually counted for the entire insert. Each treatment

group was normalized to the EBM-2 negative control and the experiment was repeated three separate times with the same conditioned medium (n=5-6) on PEC isolated from 3 patients. The relative value for each conditioned medium sample obtained in the separate assay runs was then averaged and are displayed in the figures.

5.3.6 Endothelial Cell Proliferation Assay

PEC were trypsinized and 10,000 cells per well were seeded in 96 well plates in quadruplicate for each treatment in EBM-2+ 0.5% FCS and incubated for 4 hours at 6% O_2 5% CO_2. After 4 hours a single well from each treatment group was fixed with methanol for 10 min to serve as a time zero baseline cell number. Meanwhile medium was changed to FcCM supplemented with 0.5% FCS and antibiotics, EBM-2+0.5% FCS (negative control), or EGM-2MV (positive control) in all remaining wells (in triplicate). Cells were then incubated for 48 hours. All remaining wells were then fixed with methanol and all wells including the time zero control were stained with DRAQ5 (Biostatus; Leicestershire, UK) for 1 hour. The entire plate was then scanned on the Licor Odyssey. Integrated intensity values obtained for the time zero baseline cell number wells were averaged and all other wells were normalized to this baseline cell number to represent the relative number of cells compared to time zero. Values were normalized to the EBM-2 negative control and proliferation assays were repeated with conditioned medium collected from the same placentas (n= 4-5 for each group) three separate times on PEC isolated from 3 patients. The relative value for each conditioned

medium sample obtained in the separate assay runs was then averaged and is displayed in the figures.

5.3.7 Endothelial Cell Tubule-like Structure Formation Assay

Growth factor-reduced Matrigel (GFR-Matrigel; BD) was thawed overnight on ice and plated at 45 µL/well in a 96 well plate. The plate was then incubated for 30 min at 6% O_2 5% CO_2. Meanwhile PEC were trypsinized and re-suspended in EBM-2+1%FCS with antibiotics and 10,000 cells per well were then seeded on the solidified GFR-Matrigel in triplicate for each treatment. The plate was then placed back into the incubator for 1 hour. Conditioned medium or EBM-2 was made to 1% FCS plus antibiotics and after 1 hour experimental medium, including an EGM-2MV positive control, was added. The plate was incubated for 6 hours at 6% O_2 5% CO_2 and all wells were then fixed with 4% pfa. Duplicate images were captured for each well at 5x magnification on a Leica DMIRB microscope equipped with a Q-imaging Retiga EX camera using Openlab 4.0.2 software (PerkinElmer). Images were analyzed using Image J and total tubule-like structure number was counted and length of each tubule was measured. These values were then normalized to the EBM-2+ 1% FCS negative control for each assay run. The assay was repeated three separate times with the same conditioned medium (n=3-4) on PEC isolated from 3 patients. The relative value for each conditioned medium sample obtained in the separate assay runs was then averaged and is displayed in the figures.

5.3.8 Angiogenic Factor Proteome Array

As a way to generate hypotheses about which pro- and anti-angiogenic factors were differentially regulated in fibrocyte-like cells from normal and IUGR placentas and under different oxygen conditions FcCM was produced, using the same methods presented above, from a single normal patient (patient #10) and a single IUGR patient (patient #2) (see Table 5-1). FcCM from these patients produced under normoxia and chronic hypoxia were assessed in a proteome profiler human angiogenesis array (R&D Systems; Minneapolis, MN) according to the manufacturers instructions. Data was obtained on the presence of 43 different pro- and anti-angiogenic factors and the relative expression in each group was compared. Data is presented in section 7.2 (Table 7-1).

5.3.9 Measurement of Pro- and Anti-Angiogenic Growth Factors

Specific growth factors were selected for further investigation on the basis of an observed differential expression pattern in the proteome array or a known importance in placental angiogenesis from the literature. Growth factor concentrations were measured by ELISA for IL-8 (Raybiotech; Norcross, GA), endocrine gland-vascular endothelial growth factor (EG-VEGF), activin-A, and vascular endothelial growth factor-A (VEGF) (R&D Systems) in six normal FcCM and 5 IUGR FcCM. Results were normalized to the total protein content of the wells from which the medium was collected from. Protein concentrations were measured by the BCA protein assay. IL-8 levels of 4 normal FcCM samples were found to be above detection limits for

the assay despite a 1:4 dilution, thus values for these samples were represented as the highest detectable level for the assay and normalized as for the other samples.

Pigment epithelial derived factor (PEDF) and soluble Flt-1 (sFlt) levels were determined by western blot analysis of concentrated conditioned medium. Centrifuged and filtered conditioned medium was immediately concentrated using Amicon Ultra centrifugal units (30kDa cutoff; Millipore) and frozen at -80^{o}C until needed. 24μg of protein was loaded onto a 9% SDS-PAGE gel (PEDF) or a 6% SDS-PAGE gel (sFlt-1) and then transferred onto a nitrocellulose membrane. The blots were then probed with anti-PEDF (Santa Cruz Biotechnology; Santa Cruz CA) or anti-Flt-1 (R&D Systems) overnight at 4^{o}C. After repeated washes the blot was probed with anti-goat far fluorescent-tagged secondary antibody for 1 h at room temperature. After repeated washes the blots were visualized using the Licor Odyssey. PEDF and sFlt were normalized to a running standard control that was run on each blot and corrected by the concentration factor and to the total amount of protein in the wells the conditioned medium was produced in.

5.3.10 Statistical Analysis

Statistical tests utilized for individual experiments are detailed in the figure legends. A two-way ANOVA with a Bonferroni post-hoc test was used to analyze the angiogenesis assays and to identify differences in growth factor expression between the groups. All statistics were carried out using Prism 5.0 software and a $p<0.05$ was considered significant.

5.4 Results

5.4.1 Patient Characteristics

Fibrocyte-like cells were isolated from placentas collected from 7 pregnancies complicated with IUGR and 10 normal pregnancies. Patient characteristics for all collected samples are presented in Table 5-1.

5.4.2 FcCM induced PEC migration, proliferation, and tubule-like structure formation

FcCM produced under all conditions stimulated PEC migration (Figure 5-1a). Chronic hypoxia did not have a significant effect on PEC migration in normal or IUGR FcCM, but IUGR FcCM stimulated significantly more migration than control. In contrast, FcCM collected from both normal and IUGR fibrocyte-like cells under normoxia and chronic hypoxia failed to stimulate PEC proliferation (Figure 5-1b).

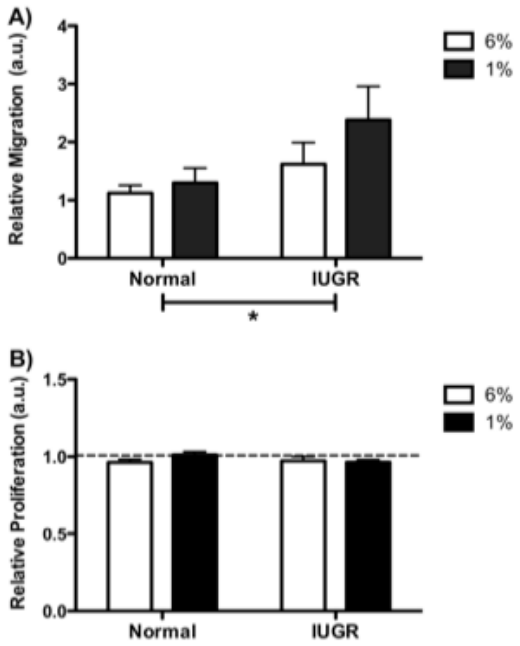

Figure 5-1: FcCM collected from IUGR fibroctye-like cells stimulates increased PEC migration compared to normal FcCM but fails to stimulate PEC proliferation

A) PEC migration; mean +/- SEM; Two-way ANOVA with Bonferroni post-hoc analysis; *= $p<0.05$; n=5-6; a.u.=arbitrary units B) PEC proliferation; mean +/- SEM; n=4-5.

Patient	Fetal Diagnosis	Birth Weight (g)	Birth Weight Percentile	Gestational Age (weeks)	Umbilical Artery Wave Form	Maternal Complications	Sex	Delivery Method	Maternal Age	Gravidity	Placental Weight (g)
1	IUGR	1390	5-10	32.86	AED/ARED		M	Cs	22	1	202
2	IUGR	700	5-10	27.14	ARED		M	Cs	28	2	102
3	IUGR	1590	<3	35.14	PED		M	Cs	33	2	269
4	IUGR	430	<3	24.57	ARED	PE, HELLP	M	Cs	22	1	156
5	IUGR	2440	<3	39.29	n/c	PE	M	Cs	22	1	355
6	IUGR	2670	5-10	38.00	n/c		M	Vd	18	1	469
7	IUGR	1070	<3	32.14	n/c		M	Cs	24	1	187
8	Norm	3280	10-50	39.14	n/c		M	Cs	36	3	n/c
9	Norm	3670	50-90	39.00	n/c		M	Cs	28	2	n/c
10	Norm	3290	50-90	38.43	n/c		F	Cs	42	3	n/c
11	Norm	3430	50-90	39.57	n/c		M	Cs	32	2	n/c
12	Norm	3600	50-90	38.71	n/c		F	Cs	31	2	n/c
13	Norm	3110	10-50	39.14	n/c		F	Cs	32	1	n/c
14	Norm	4440	>97	39.29	n/c		M	Cs	29	2	n/c
15	Norm	3250	50-90	37.71	n/c		M	Cs	32	2	n/c
16	Norm	3170	50-90	38.57	n/c		M	Cs	34	5	n/c
17	Norm	3090	50-90	38.14	n/c		M	Cs	30	4	n/c

Table 5-1: Patient Characteristics
n/c= not reported; Norm= normal; AED= absent end diastolic flow; ARED= absent or reversed end diastolic flow; PED= persistent end diastolic flow; PE= preeclampsia; HELLP= HELLP syndrome; Cs= caesarean section; Vd= vaginal delivery

FcCM has previously been shown to stimulated PEC and HUVEC tubule-like structure formation on GFR-Matrigel (19). FcCM produced by IUGR fibrocyte-like cells stimulated formation of ~22% fewer tubule-like structures than FcCM from normal fibrocyte-like cells (Figure 5-2a). IUGR-FcCM also resulted in an increased tubule-like structures length by ~7% compared to normal FcCM (Figure 5-2b). Thus IUGR-FcCM stimulated the formation of significantly fewer but longer tubules overall. There was no difference in the number of tubule-like structures or the tubule-like structure length when PEC were treated with FcCM produced under chronic hypoxia.

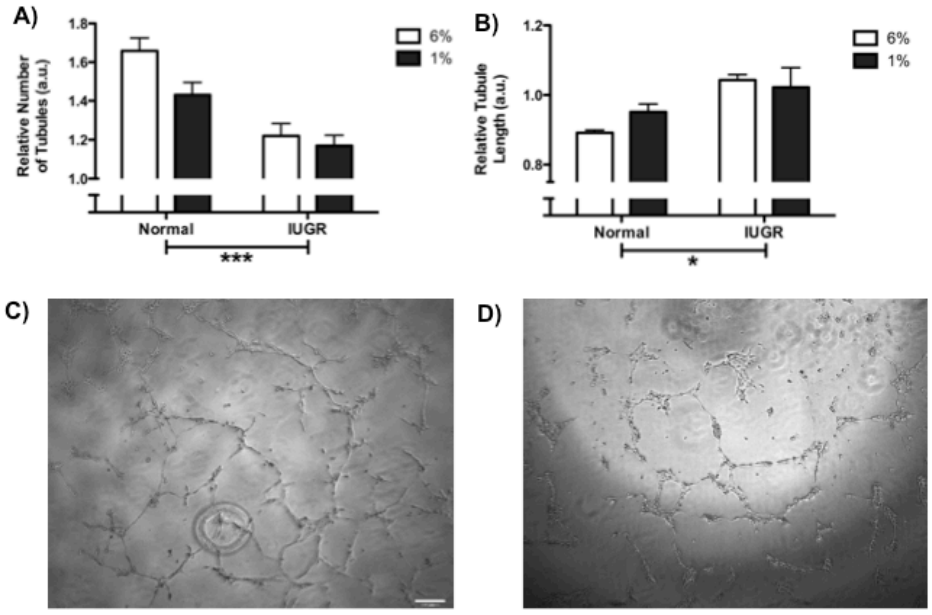

Figure 5-2: IUGR FcCM has a reduced ability to stimulate PEC tubule-like structure formation
A) Summary graph of relative number of tubule-like structures; B) Summary graph of relative tubule-like structure length; mean +/- SEM; two-way ANOVA with Bonferroni post-hoc analysis; ***=$p<0.001$; *=$p<0.05$; n=3-4; a.u.=arbitrary units. Representative images of tubule-like structures C) Normal FcCM 6% O_2; D) IUGR FcCM 6% O_2.

5.4.3 Characterization of differential angiogenic factor expression in FcCM

The amount of the pro-angiogenic factors IL-8, VEGF, and EG-VEGF in FcCM were examined by ELISA. IL-8 was found to be significantly lower in the FcCM from IUGR samples (3-7 fold

lower) but no effect of chronic hypoxia was observed (Figure 5-3a). VEGF levels were not significantly different between normal and IUGR FcCM but chronic hypoxia was found to result in a three-fold increase in VEGF compared to FcCM produced under normoxia (Figure 5-3b). No differences were found between any of the groups for EG-VEGF (Figure 5-3c).

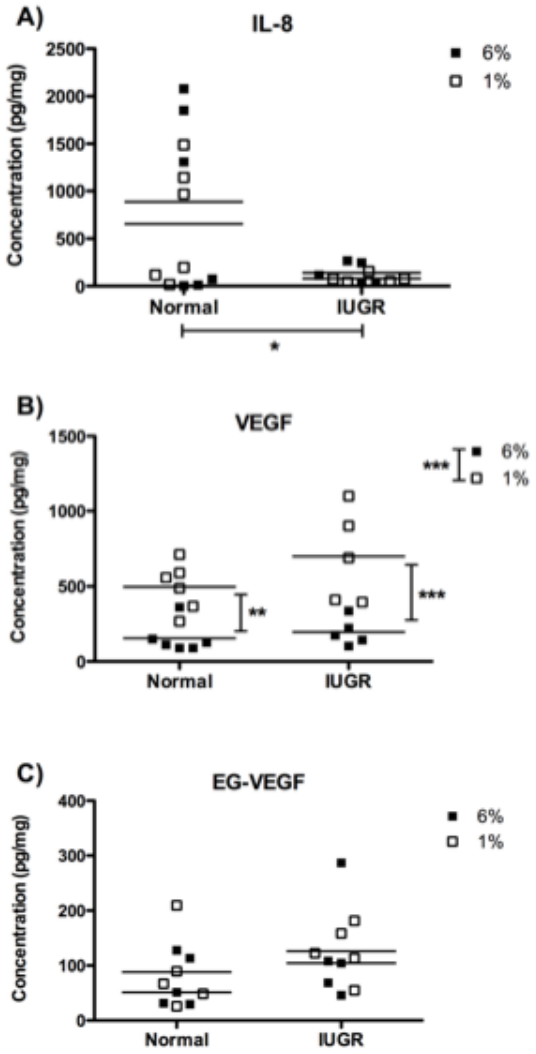

Figure 5-3: FcCM from IUGR fibrocyte-like cells has decreased levels of pro-angiogenic factors compared to normal FcCM
A) Summary graph of IL-8 measured by ELISA; two-way ANOVA with Bonferroni post hoc analysis; line represents mean; n=5-6; *=$p<0.05$; B) Summary graph of VEGF-A measured by ELISA; n=5-6; **=$p<0.01$, ***=$p<0.001$; C) Summary graph of EG-VEGF measured by ELISA; n=5.

The anti-angiogenic factors activin-A, PEDF, and sFlt were also examined. Activin-A was found to be ~3 fold higher by ELISA in IUGR FcCM but no effect of chronic hypoxia was observed (Figure 5-4a). PEDF was also found to be in ~5 fold higher abundance in IUGR FcCM at 6% O_2, and was found in significantly decreased levels (~5-8 fold) in FcCM produced under chronic hypoxia by western blot analysis (Figure 5-4b). Chronic hypoxia increased the levels of sFlt-1 found in FcCM by western blot analysis by ~3 fold, but there was no difference in sFlt levels between normal and IUGR FcCM (Figure 5-4c).

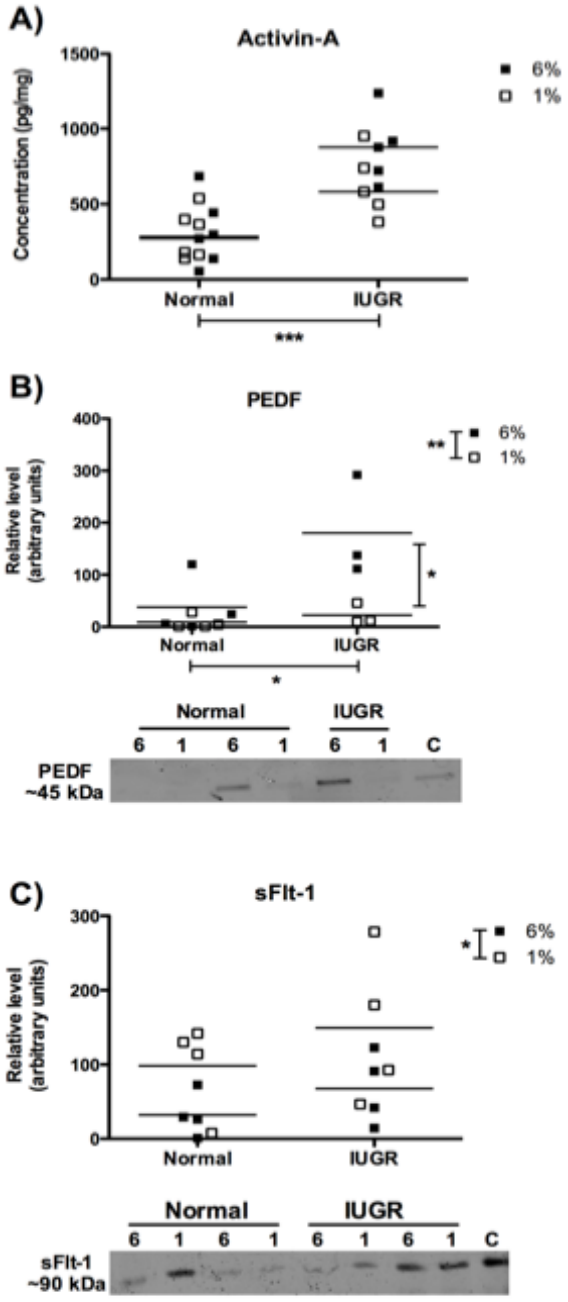

Figure 5-4: FcCM from IUGR fibrocyte-like cells has increased levels of anti-angiogenic factors compared to normal FcCM

A) Summary graph of activin-A measured by ELISA; two-way ANOVA with Bonferroni post hoc analysis; line represents mean; n=5-6; ***=p<0.001; B) Summary graph of PEDF western analysis and a representative western blot; n=3-4; 6=6% O_2; 1= 1% O_2; C= loading control; **=p<0.01, *=p<0.05; C) Summary graph of sFlt-1 western analysis and a representative western blot; n=4; *=p<0.05.

5.5 Discussion

In this study we have found that FcCM from IUGR placentas have a reduced ability to stimulate endothelial cell tubule-like structure formation and an increased ability to stimulate endothelial cell migration compared to fibrocyte-like cells from normal placentas. It was found that IUGR FcCM contained reduced levels of the pro-angiogenic factor IL-8 and increased levels of the anti-angiogenic factors activin-A and PEDF. Thus fibrocyte-like cells isolated from IUGR placentas have an altered ability to stimulate angiogenesis *in vitro*. This implies that there are inherent differences in the behavior of the same cell type in placentas that result in reduced fetal growth and those that allow for adequate fetal growth. A difference in the ability of conditioned medium collected from placental explants from normal and IUGR placentas to stimulate angiogenesis *in vitro* has been previously shown by Padavala et al (20). Padavala et al. did not see a difference in the ability of conditioned medium from IUGR explants to stimulate HUVEC tubule-like structure formation at high oxygen tension (20% O_2) but saw a reduction in the tubule-like structure length alone when conditioned medium was collected from explants cultured at 3% O_2. Thus the differential ability of normal versus IUGR placental cells to stimulate angiogenesis required a low oxygen environment to be observed. In our study, specifically examining fibrocyte-like cells, IUGR FcCM stimulated formation of fewer tubule-like structures under both normoxia (6% O_2) and chronic hypoxia (1% O_2) and IUGR FcCM simulated an increased tubule-like structure length which was only significant at 6% O_2. Thus both Padavala et al. and our study show that normal and IUGR placental cells have differential abilities to stimulate angiogenesis *in vitro*. Conditioned medium collected from placental explants is more representative of the soluble factors produced by the syncytiotrophoblast therefore it

appears that a dysfunctional ability to stimulate angiogenesis is present in two different populations of cells that are involved in controlling placental angiogenesis.

A secondary goal of this study was to examine whether exposure to chronic hypoxia would result in a decreased ability of fibrocyte-like cells to stimulate angiogenesis *in vitro* irrespective of their isolation from normal or IUGR placentas. It was more specifically hypothesized that this would be due to an inability of the fibrocyte-like cells to produce a pro-angiogenic response to hypoxia when hypoxia was administered chronically. It was found that maintenance in chronic hypoxia did not result in any differential ability of FcCM to stimulate endothelial cell proliferation, migration, or differentiation into tubule-like structures, therefore chronic hypoxia does not result in a decreased ability to stimulate any aspect of angiogenesis under these conditions. Additionally, an increased amount of VEGF and sFlt and a decreased amount of PEDF was found in conditioned medium produced under chronic hypoxia. Hypoxia is a known inducer of pro-angiogenic factors such as: VEGF, stromal cell-derived factor-1, endothelin-1, IL-8, and EG-VEGF and the anti-angiogenic factor sFlt (21,22). In this study chronic hypoxia led to the elevation of both VEGF and sFlt. This implies that the HIF pathway is activated despite the chronic nature of the insult, for both of these factors are known to be regulated by the HIF pathway (21). Up-regulation of IL-8 and EG-VEGF reported in the literature is independent of the HIF pathway (21) and there was no effect of chronic hypoxia on the levels of either factor in our study. In this study PEDF production was also found to be controlled by hypoxia, with a reduced amount of PEDF found in FcCM produced under chronic hypoxia. Hypoxia has previously been shown to both increase and decrease PEDF expression depending on the

cell type and its regulation has been shown to be both HIF dependent and independent (23-25). Ultimately, in this study we found that although there is a significant increase in VEGF-A after chronic hypoxic treatment this increase is not able to influence PEC proliferation, migration, or tubule-like structure formation, likely due to the observed increase in sFlt levels. Thus the balance of factors found in the FcCM under chronic hypoxia are ultimately no more pro-angiogenic than FcCM produced under physiologic normoxia.

In order to try to understand the mechanisms through which IUGR fibrocyte-like cells were stimulating the reduced tubule-like structure formation and increased PEC migration we examined the abundance of several pro- and anti-angiogenic growth factors in the conditioned medium. A significant reduction in the pro-angiogenic factor IL-8 and an increase in the anti-angiogenic factors activin-A and PEDF were found. These observations help to explain the decreased tubule-like structure formation observed with IUGR FcCM but fail to explain the observed increase in PEC migration. One potential pathway that was not validated but was identified by the proteome array that could be contributing to the observed increased PEC migration to IUGR FcCM is an increased expression of moncyte chemoattractant protein-1 (MCP-1). MCP-1 has been shown to directly stimulate endothelial cell migration at lower doses than it stimulates endothelial cell sprouting in the rat aortic ring assay (500pg/mL vs. 50ng/mL) (26). Thus if low levels of MCP-1 are present in the conditioned medium it may be able to stimulate PEC migration without significantly contributing to tubule-like structure formation.

Another observation that warrants further investigation is the lack of PEC proliferation when cells were treated with FcCM. Concentrated FcCM was also tested but proliferation was not significantly improved (data not shown) thus ruling out the possibility that the concentration of growth factors in the conditioned medium was too low to stimulate proliferation. Therefore it is likely that there is some factor or group of factors blocking proliferation. EG-VEGF selectively act on the placental microvascular endothelium to stimulate proliferation, migration, and differentiation whereas it only stimulates macrovascular umbilical endothelial cell survival (27). In this study HUVEC failed to migrate towards FcCM and also failed to proliferate in the presence of FcCM (data not shown). Thus there may be additional factors, in addition to EG-VEGF, in FcCM that may have placental specific angiogenic effects. Clear characterization of the components of FcCM could therefore illuminate previously unidentified factors that are contributing to the decreased angiogenesis stimulated by IUGR fibrocyte-like cells.

Ultimately this communication has shown that there are inherent differences in the ability of normal versus IUGR fibrocyte-like cells to stimulate angiogenesis, and that a chronically hypoxic environment is not able to differentially affect the ability of fibrocyte-like cells to stimulate angiogenesis *in vitro*. Thus the differential production of angiogenic growth factors by fibrocyte-like cells may contribute reduced placental growth and ultimately to the development of IUGR.

5.6 References

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Chapter 6: General Discussion and Future Directions

As stated repeatedly throughout this thesis, the placenta is an organ of upmost importance to fetal growth that remains a massively understudied topic area in human health. With up to 10% of all births affected by placental insufficiency in the form of IUGR and/or preeclampsia and a further ~1% affected by invasive placental disorders such as placenta previa or placenta accreta, a significant impact on maternal and fetal health could be attained if treatments for this range of disorders could be established (1-4).

Importantly, there are currently no curative therapies for any of these conditions and clinical management involves close monitoring of fetal wellbeing with delivery if the fetus begins to fail or if the mother becomes dangerously ill (2,5). Much research is currently going on to identify therapies that would improve fetal growth and extend the length of gestation and there are several potential targets for treatment. Perhaps the most common target of potential therapies is to identify agents that would increase uterine blood supply and would therefore have application in both preeclampsia and IUGR. Of promise in this area is sildenafil citrate, a myometrial vasodilator, that has been used in a very small number of patients with early-onset IUGR (<25 weeks) and was shown to increase the fetal abdominal circumference (6). Another obvious target for treatments is to increase either the growth or efficiency of the placenta. Potential therapies could target

angiogenesis, trophoblast differentiation, and ST transport. Hopefully previous sections of this thesis have impressed upon you just how little is ultimately known about the control of placental growth, therefore targeted therapies towards any of these pathways require much more basic research in order to identify key regulatory pathways that could be manipulated for therapeutic benefit. The development of effective treatments necessitates a better understanding of the basic processes that control placental formation, invasion, and growth. Thus by examining the fundamental processes of trophoblast differentiation and angiogenesis this thesis makes an important contribution towards understanding key regulators of placental growth moving us closer to the development of treatments for these conditions.

6.1 Summary of the most significant findings

The first half of this thesis contains studies examining trophoblast differentiation. In Chapter 2 we provided evidence that trophoblast differentiation does not involve the caspase-independent mediator AIF concluding that trophoblast differentiation is not dependent on either known apoptotic cascade. In Chapter 3 we went on to examine whether PS externalization, another apoptosis-associated event, was involved in trophoblast differentiation. We found that PS externalization does not occur in trophoblastic cells above the basal level of apoptosis unless differentiation is stimulated with the *Coleus forskholii* plant extract forskolin. We established that PS externalization observed in primary trophoblasts when stimulated with forkolin is due to pleiotropic effects of the compound and concluded that due to the low proportion of cells with externalized PS during the differentiation process that PS externalization is unlikely to be involved in trophoblast differentiation.

The second half of this thesis was devoted to examining villous placental angiogenesis. In Chapter 4 we provided evidence of a novel population of placental stromal cells, fibrocyte-like cells, and the ability of these cells to stimulate angiogenesis. In Chapter 5 we extended this study and found that fibrocyte-like cells from IUGR placentas have a reduced ability to stimulate angiogenesis likely due to the reduced expression of pro-angiogenic factors (IL-8) and increased expression of anti-angiogenic factors (activin-A and PEDF) found in IUGR fibrocyte-like cell

conditioned medium. It was also observed that maintenance in chronic hypoxia has no effect on the ability of fibrocyte-like cells to stimulate angiogenesis.

6.2 The Involvement of the Apoptotic Cascade in Trophoblast Differentiation

Data showing evidence of the initiation of the apoptotic cascade, or more specifically the activation of the initiator caspase, caspase 8, and the externalization of the inner-membrane leaflet phospholipid PS, have jointly been used as evidence by Huppertz et al. that apoptosis is initiated early in the trophoblast differentiation process and is required for trophoblast differentiation to occur (7-12). This hypothesis has come under increasing scrutiny and evidence showing that active caspase 8 and activation of the caspase-dependent apoptotic cascade are not involved in trophoblast differentiation has been published (13-15). Additionally it has been shown that PS efflux during trophoblastic differentiation occurs independently of any other markers of apoptosis and is therefore an apoptosis-independent event, at least in the Bewo trophoblastic cell line (15). As we have produced evidence in our lab that trophoblast fusion occurs independently of caspase-dependent apoptosis, our studies on trophoblast differentiation in this thesis were focused on examining whether caspase-independent apoptosis and PS efflux are involved in trophoblast differentiation. Both of these studies provide evidence that caspase-independent apoptosis and PS externalization are not required for trophoblast differentiation. We conclude based on the studies presented here and our previous work, that trophoblast differentiation does not involve any known type of apoptosis. Thus, we propose that future research on

trophoblast differentiation should focus on cell signaling pathways that are not involved in the process of apoptosis.

6.3 Future Directions in Trophoblast Differentiation

6.3.1 Guanine-nucleotide Exchange Factors

An interesting future direction for the field of trophoblast differentiation would be to investigate cellular signaling pathways and proteins that have been shown to be involved in other cellular systems in which fusion is occurring, such as myoblast and macrophage fusion and HIV or cancer induced syncytia formation. Specifically the GTPase Rac1 and guanine-nucleotide exchange factor (GEF) dock180 play an important role in fusion of both myoblasts and macrophages and seems a worthy avenue of future research in the trophoblast (16). It remains to be established whether dock180 is expressed in trophoblasts, but Rac1 has previously been shown to play important roles in EVT migration (17,18). Rac1 is involved in controlling the formation of lamellipodia and stress fibers in other cell types thus it may be involved in the rearrangement of the cytoskeleton during trophoblast differentiation (16,18).

Another interesting GEF that has recently been shown to be involved in trophoblast fusion is RhoE (19). RhoE was found to be up-regulated by cAMP treatment of Bewo, with the highest abundance of RhoE coinciding with the onset of fusion, and RhoE knockdown resulted in decreased fusion (19). This GEF is known to be associated with the negative regulation of RhoA, inhibits stress fiber formation in fibroblasts and neurons, and is also involved in cell cycle inhibition (20). Importantly RhoE knockdown did not lead to a decrease in functional differentiation, thus the

elucidation of the complete signaling cascade associated with RhoE up-regulation will allow for illumination of fusion and functional differentiation specific pathways (19). These observations were made entirely in the Bewo cell line, though, so confirmation of similar results in primary cell is an essential first step.

6.3.2 Wnt/β-Catenin Signaling Pathway

Another exciting future direction for the field is the elucidation of the ligand, the receptor, and the exact signaling pathway through which Wnt/β-catenin pathway is controlling GCM1 expression and therefore trophoblast fusion. The Wnt/β-catenin signaling pathway is very complex with 19 Wnts and 15 receptors and many more co-receptors identified (21). Due to the importance of Wnt in essential processes such as embryonic patterning, stem cell differentiation, and its emerging role in cancer, much intensive research has been done in this field giving placentologists the benefit of many papers characterizing this convoluted pathway from which to begin studies (21). As Matsuura et al. (2011) indicated in the discussion of their paper identifying the Wnt/β-catenin components BCL9L, β-catenin, and TCF4 as regulators of GCM1 expression, the phenotype of several published knockout mice have already given us clues as to which Wnt receptors and pathway component may be involved in trophoblast differentiation (22). Matsuura et al. specifically mention the likely involvement of agonist R-spondin 3 and the Frizzled 5 receptor based on the phenotype of *R-spondin 3* and *Frizzled 5* knockout mice. R-spondin 3 does not bind to Frizzled 5 though and the R-spondin 3 interaction with the Wnt/β-catenin pathway involves several other key proteins. R-spondins are secreted proteins that

have recently been shown to interact with the Leu-rich-repeat-containing G protein coupled receptors 4-6 (LGR) (21). When R-spondins interact with their receptor they form a complex with zinc and RING finger 3 (ZNRF3), and when ZNRF3 it is not complexed with R-spondin/LGR it ubiquitinylates Frizzled receptors targeting them for degradation. Thus when R-spondin binds to LGR4, 5 or 6 it leads to an increased accumulation of Frizzled on the cell surface and enhanced Wnt signaling (see Figure 6-1).

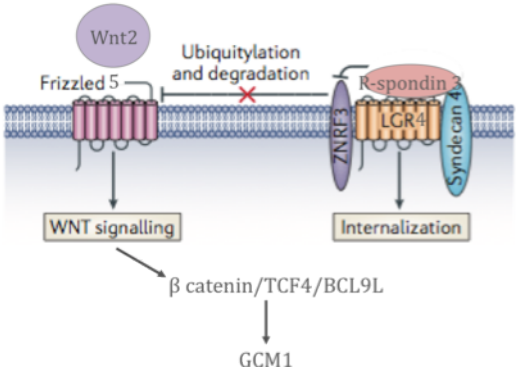

Figure 6-1: Wnt/β-catenin pathway components potentially involved in trophoblast fusion as indicated by their knockout mouse phenotype
R-spondin 3 binding to the Leu-rich-repeat-containing G protein coupled receptor 4 (LGR4) may block zinc and RING finger 3 (ZNRF3) mediated ubiquitylation of the Frizzled 5 receptor therefore allowing an increased abundance of Frizzled 5 to be found on the cell surface to bind the potential ligand Wnt2. Wnt2/Frizzled 5 interaction would lead to β-catenin dependent signaling and increased expression of glial cell missing-1 (GCM1) ultimately resulting in trophoblast fusion. β-catenin, T-cell factor 4 (TCF4), B-cell lymphoma like 9 ligand (BCL9L), and GCM1 are the only pathway components for which direct evidence of involvement in trophoblast fusion has been shown. *Modified from: Niehrs C. The complex world of WNT receptor signaling. Nat. Rev. Mol. Cell Biol. 2012; 13: 767-79.*

The *Frizzled 5* knockout mouse is embryonic lethal at embryonic day 10.75 due to defective vasculogenesis in the yolk sac and placenta (23). It is noted that there is no difference in the number of trophoblastic cells present in the knockout placentas at embryonic day 10.75 but a complete absence of fetal vascular invasion into the deeper labyrinth is observed (23). Thus due to the timing of the embryonic lethality,

a prominent placental phenotype of the knockout, and an established high level of expression of Frizzled 5 on isolated CT and in human placental samples, further investigation into the role this receptor may play in fusion is tantalizing (23,24). Wnt2 is a likely candidate for interaction with the Frizzled 5 receptor based again on the phenotype of the already characterized *Wnt2* knockout mouse. *Wnt2* knockout mice survive to birth but display a ~33% decrease in weight and by embryonic day 14.5 knockout placentas displayed a significantly reduced number of fetal capillaries in the labyrinth zone, though no alterations in trophoblastic cells are mentioned (25). Wnt2 is strongly expressed in both first and third trimester isolated CT, whole placenta, and in first trimester fibroblasts, again indicating an exciting potential avenue to explore in trophoblast differentiation (24).

A significant decrease in the expression of GCM1 is noted in *R-spondin 3* knockout mice placentas along with an absence of vascular invasion of the labyrinth zone at embryonic day 10.5, thus displaying the most compelling linkage directly to trophoblast fusion of any of the knockout mice presented (26,27). The R-spondin 3 receptor LGR4 knockout mice also have an interesting phenotype as the pups that survive to term are growth restricted by 14%, but unfortunately no examination of the placental phenotype was reported (28). LGR4 expression has also been reported to be high in the human placenta (29).

Obviously much evidence is already available from the mouse literature as to the many different Wnt/β-catenin pathway components that may be important for

trophoblast differentiation and experiments utilizing human tissue and cells to confirm these observations are essential.

6.3.3 Phosphatidylserine Externalization and Trophoblast Fusion

Chapter 3 of this thesis specifically examined the involvement of PS externalization in trophoblast fusion. Previous studies found that large amounts of externalized PS were observed in Bewo and JAR concomitant with cellular fusion after treatment with forskolin (15,30). Additionally, a monoclonal anti-PS antibody blocked forskolin induced cellular fusion in JAR (15,30). Together these results were used to conclude that PS externalization is required for trophoblastic cellular fusion. In contrast, we found that PS externalization was not occurring in primary trophoblasts and did not see extensive PS externalization in forskolin treated Bewo. Our study indicated that large amounts of PS externalization are unlikely to be involved in trophoblast fusion, but we were unable to examine whether small amounts of externalized PS at the sites of membrane fusion maybe involved. We utilized a commercial anti-PS antibody and performed differentiation experiments in the presence of the antibody and found no inhibition of fusion. Unfortunately, we did not have access to the original anti-PS antibody utilized with JAR cells so it is difficult to ascertain whether our experimental conditions were adequate to block localized PS externalization, thus future experiments examining whether potentially rapid and localized PS externalization is occurring during fusion are required.

6.3.4 Usage of Forskolin as a Differentiation Agent and Trophoblast Differentiation Models

The evidence presented in Chapter 3 highlighting the pleiotropic effects of forskolin on trophoblasts indicates that this compound should be utilized cautiously in future studies examining trophoblast differentiation. The field would do best to use multiple different conditions in a single study, such as treatment with Br-cAMP or activin-A, as well as culture conditions where high levels of spontaneous differentiation occur (such as (31)) to corroborate results obtained with forskolin on isolated cells and cell lines. It would also be beneficial to develop models utilizing mixtures of factors and substrates to treat isolated cells, for treatment with a single factor is less likely to involve the diverse pathways that would be stimulated when multiple signals from many different factors must be integrated by the cells. Alternatively, an interesting potential protocol could involve stimulating isolated cell differentiation with the conditioned medium of placental explants. This complex mixture would, in theory, be quite representative of the soluble factors present *in vivo* (although more representative of the apically than basally secreted factors from the ST) and thus an interesting model for examining the molecular pathways involved in fusion and functional differentiation. Experiments utilizing explant-conditioned medium would be very interesting to compare to the results of Gerbaud et al. (2011) (32) who developed a model using conditioned medium from placental stromal cells to stimulate trophoblast fusion. Experiments utilizing both models would be able to highlight specific factors that are provided by each cellular compartment and may illuminate specific roles of the intact ST and stromal cells. It would also be very interesting to utilize fibrocyte-like cell conditioned medium,

which was used to examine angiogenesis in Chapters 4 & 5, in trophoblast fusion experiments for they likely represent a different population of stromal cell than those utilized by Gerbaud et al. and additional factors involved in differentiation may be discovered. It would also be interesting to repeat the experimental design utilized in Chapter 5 to see whether FcCM from IUGR fibrocyte-like cells stimulated similar levels of trophoblast differentiation as FcCM from normal placentas and whether production of the medium in chronic hypoxia had any effect on the amount of differentiation that FcCM may induce.

6.4 Trophoblast Differentiation Study Limitations

Some limitations exist with regards to the trophoblast differentiation studies completed in this thesis. The major limitation is the utilization of isolated cell culture for all of the studies. Studying an isolated pure population of cells allows us to examine cellular responses at the level of a single cell type in order to not have interfering responses of other types of cells, but the microenvironment in which the cells are maintained and studied in is therefore different from the *in vivo* situation and cellular responses may not mimic the *in vivo* response. However, the placenta is a tissue that is not possible to study *in vivo* due to its inaccessible nature and the danger of interventions and investigations to both the mother and the developing fetus, thus necessitating tissue culture. Additionally, commonly utilized rodent models have similar molecular regulation to the human placenta, but different placental structure and are therefore not ideal for examining trophoblast differentiation (33). The strengths of our trophoblast differentiation studies are that the primary cells were never passaged, therefore excluding the effects of long-term culture on cells and avoiding the issue of using transformed cell lines.

An important detail to consider for both of the trophoblast differentiation studies is the atmospheric culture conditions that the experiments were performed under. The AIF study was performed exclusively at 8% O_2 5% CO_2 whereas the PS study was conducted under standard culture conditions (20-21% O_2 5% CO_2). Primary

trophoblast susceptibility to apoptosis has previously been shown to increased at the extremes of oxygen tension used in cell culture (21% O_2 and 1% O_2) with a marked protection against apoptosis at more physiologic oxygen levels (34,35). Therefore, by performing our PS experiments at room air (20-21% O_2) the results were obtained in an environment where apoptosis was slightly more likely to occur. This oxygen tension was chosen for these experiments in order to replicate the conditions that had previously been shown to reveal PS externalization during differentiation (15,30) and since the levels of apoptosis that we observed in the study peaked at ~3% it is unlikely that utilizing this environment had significant effects on our results.

6.5 The Role of Fibrocyte-like cells in Placental Angiogenesis

Chapter 4 of this thesis presented the identification and angiogenic potential of a novel population of peripheral placental stromal cells that we designated as fibrocyte-like cells. These cells were found to be localized primarily within the terminal and mature-intermediate villi and could be identified by a combination of hematopoietic lineage (CD115, CD14) and fibroblastic markers (TE-7). *In vitro* these cells would be isolated from a $CD45^+$ population, though they eventually become $CD45^-$ indicating that at least one pathway for their differentiation is from hematopoietic progenitors or through trans-differentiation from Hofbauer cells. The conditioned medium from fibrocyte-like cells was found to stimulate PEC tubule-like structure formation on GFR-Matrigel and it was concluded that fibrocyte-like cells are likely to play a role in placental angiogenesis.

In Chapter 5 we extended the study to examine whether fibrocyte-like cells from IUGR placentas had the same ability to stimulate angiogenesis as those derived from normal pregnancies. It was found that the IUGR fibrocyte-like cells have a reduced ability to stimulate PEC tubule-like structure formation and an increased ability to stimulate PEC migration. IUGR FcCM was found to contain reduced levels of the pro-angiogenic factor IL-8 and increased levels of the anti-angiogenic factors activin-A and PEDF and it was concluded that the reductions in tubule-like structure formation and increased migration may be a result of this differential expression.

6.6 Future Directions for Fibrocyte-like Cell Studies

6.6.1 Identification of Specific Cellular Markers

Several interesting future studies could be completed to follow up on some of the questions revealed by this work. An obvious future direction is the identification of a specific cellular marker for fibrocyte-like cells so that they could easily be identified and separated from the other fibroblastic cells of the placenta. With increased awareness that cells labeled as fibroblasts likely represent a heterogeneous mixture of different populations of cells and with intensive research currently in the field of cancer biology examining the role fibroblastic cells play in tumor biology the discovery of new specific markers for different fibroblastic cell-types is likely. Development of antibodies for novel fibroblastic markers are therefore likely to be available in the near future and can then be able to be applied to the fibroblastic stroma of the placenta.

6.6.2 Identification of Different Placental Cells Using Cell Surface Proteomics

Identifying the specific cell types that make up the human and rodent placentas is an important future direction for the field of placentology that is an obvious extension of the work presented in Chapters 4 and 5. With the advent of cell surface proteomics, identification of the individual populations of cells within the placentas is now possible. This technology is already being used to identify the specific populations of cells that are found during embryonic stem cell differentiation (36). Some initial work identifying specific markers for placental endothelial cells and trophoblasts in mice has been published (37) and the extension of studies similar to

this one to human tissue will be very powerful. Parallel studies examining the different populations of cells within the human and murine placenta would help us to better understand the equivalent cell types to allow for more effective future research using the murine model. Another exciting study that could be carried out with this technology would be to examine what type of cells are found within the placenta at different points in gestation. As the technology utilizes cell surface proteins, the other obvious direct application would be the development of cell isolation protocols for the different cell types. Studies with similar designs as Chapter 5 could be carried out to identify the differences between placental cells from IUGR and PE pregnancies and even whether the same types of cells are present in the placenta in IUGR and PE.

6.6.3 Identification of Novel Tissue Specific Angiogenic Factors:

Another important future direction from these studies would be the extension of one of the most intriguing results, namely the observation that PEC, but not HUVEC were found to migrate towards FcCM. Tissue specific angiogenic mediators such as EG-VEGF exist and control different aspects of angiogenesis (38,39). EG-VEGF has been shown to significantly stimulate PEC migration, tubule-like structure formation, and proliferation, but HUVEC do not respond and EG-VEGF only seems to stimulate survival in this macrovascular cell-type (38). Additionally, FcCM did not stimulate proliferation in either HUVEC or PEC in our study, but did stimulate both migration and tubule-like structure formation. No differences were observed in the amount of EG-VEGF found in normal versus IUGR FcCM thus it is unlikely to be contributing to the decreased tubule-like structure formation and increased PEC

migration observed with IUGR FcCM. It is therefore likely that some other angiogenic factor, or group of factors, is selectively acting on the PEC to stimulate migration. Thus further characterization of the factors in FcCM may reveal novel tissue specific angiogenic mediators.

6.6.4 Examination of Known Angiogenic Factors with PEC

These results also highlight the importance of using the appropriate endothelial cells when examining placental angiogenesis. Studies that exclusively utilize HUVEC may ultimately come to very different conclusions than those that would be obtained if PEC were used. Future studies examining the effects of known angiogenic mediators (such as VEGF-A, b-FGF, PlGF) should also be carried out with PEC in order to understand whether these important factors act through the same mechanisms previously identified in HUVEC and other endothelial cell types. Studies that simply link the expression of specific pro- and anti-angiogenic factors to function would also be better served through examination of the effects of the factor on PEC directly, for unexpected effects may be observed.

Finally, the proteome assay that was run as a hypothesis generating mechanism (results presented in Appendix B) identified many pro- and anti-angiogenic growth factors that may play important roles in placental angiogenesis. Strong relative expression of several factors including angiogenin, angiostatin, coagulation factor III, hepatocyte-growth factor, insulin-like growth factor binding protein-1,-2, and-3, macrophage inflammatory factor-1 alpha, pentraxin-3, platelet- derived endothelial cell growth factor, thrombospondin-1, and urokinase plasminogen activator were

observed and the direct angiogenic effects of all of these factors have never been examined in the placenta.

6.7 The Role of Chronic Hypoxia in the Deficient Angiogenic Response of IUGR Placentas

One of the main hypotheses about how IUGR develops holds that due to shallow trophoblastic invasion the placenta develops in a hypoxic environment and that hypoxia results in the villous malformation and malfunction observed in the established condition resulting in suboptimal growth of the fetus. Hypoxia is a well-established stimuli for angiogenesis, thus the paradoxical situation in IUGR where angiogenesis is clearly deficient requires investigation (40). We put forth the novel hypothesis that chronic long-term unresolved hypoxia would ultimately result in cessation of angiogenesis due to a focus on cell survival in the sub-optimal environment instead of sustaining the classical pro-angiogenic response for prolonged periods. Contrary to our hypothesis the classical HIF-dependent pro-angiogenic response was observed in fibrocyte-like cells that had been exposed to chronic hypoxia. Namely there was a classical increase in VEGF found in FcCM and a concomitant increase in sFlt-1 levels, though the abundance and localization of HIF-1α was not directly queried. Also in contradiction to our hypothesis, FcCM produced in cells exposed to chronic hypoxia elicited the same amount of angiogenesis *in vitro* as FcCM produced at physiologic normoxia. Thus it appears that despite responding to exposure to chronic hypoxia by changing the production of angiogenic factors the balance of the factors elicits the same degree of angiogenic response from the PEC. As we have only examined the response of fibrocyte-like cells to chronic hypoxia it remains to be seen whether our hypothesis may hold true with the other placental

cell types involved in angiogenesis or when multiple cell types are cultured together.

6.8 Future Directions for the Role of Chronic Hypoxia in the Deficient Angiogenic Response of IUGR Placentas

6.8.1 Chronically Hypoxic PEC

Preliminary data presented in Appendix A showed that when PEC are maintained at chronic hypoxia and treated with FcCM produced in a chronically hypoxic environment (in this pilot study 3% O_2 compared to the 1% O_2 utilized in Chapter 5) that there was a trend towards reduced tubule-like structure formation ($p=0.07$). In contrast to the study presented in Chapter 5 we could not find any significant changes in either pro- or anti- angiogenic growth factor production in the chronically hypoxic FcCM in the pilot study.

When the results of the pilot study and those in Chapter 5 are considered together they indicate that a titillating future direction would be to further examine the effects of FcCM produced under chronic hypoxia on PEC that are maintained in a chronically hypoxic environment themselves. This more physiologic situation was the design used in the pilot study, but with this design it would have been very difficult to ascertain whether differences in angiogenesis were due to changes in the amount of soluble factors produced by fibrocyte-like cells, a change in sensitivity by the PEC, or any combination of both situations. As the focus of the thesis was on fibrocyte-like cells and not the endothelium we chose to continue with the experiments presented in Chapter 5 where only the fibrocyte-like cells were

exposed to chronic hypoxia. If experiments where both the PEC and fibrocyte-like cells are maintained in chronic hypoxia were going to be continued, an essential first step would be to conduct a detailed study examining the angiogenic response of PEC after maintenance in chronic hypoxia. Once the behavior of PEC in chronic hypoxia was established research examining both PEC and fibrocyte-like cells exposed to chronic hypoxia could be undertaken. A complementary study examining the angiogenic potential of IUGR PEC, and their response to chronic hypoxia would also be an exciting direction.

6.8.2 Varying Exposure to Hypoxia

Another logical extension of the work presented in Chapter 5 would be to examine the production of angiogenic factors by fibrocyte-like cells after varying lengths of time in a hypoxic environment. These experiments would reveal whether changes in the quantity and mixture of angiogenic factors produced by fibrocyte-like cells occurs when they are exposed to hypoxia for varying periods of time. These experiments would more directly test our hypothesis that the pro-angiogenic response elicited by hypoxia is reduced when hypoxia becomes chronic in nature.

6.8.3 The Response of IUGR+ARED vs. IUGR+PED to Chronic Hypoxia

As discussed in Chapter 1 differences in the appearance of the vasculature in IUGR+ARED and IUGR+PED placentas have been shown and it has previously been hypothesized that these differences were due to relative placental hyperoxia in IUGR+ARED (41). An alternative hypothesis is that instead of exposure to different levels of oxygen, that the cells in a placenta that goes on to develop IUGR+ARED

react to chronic hypoxia differently than those that develop IUGR+PED. Therefore another attractive future direction for this work would be to examine if fibrocyte-like cells isolated from IUGR+ARED react differently in terms of their angiogenic factor production than fibrocyte-like cells from IUGR placentas with PED both at physiologic oxygen concentrations and after maintenance in chronic hypoxia.

6.9 Fibrocyte-like Cell Study Limitations

Limitations exist for the work presented in Chapters 4 and 5, the first of which being the extended nature of the culture of fibrocyte-like cells and PEC. Extended cell culture has been shown to result in a decreased proliferation, morphological changes, reduced ability of cells to differentiate, and loss of stem cell marker expression (42-45). To minimize this limitation fibrocyte-like cells were only used for experiments below passage 10 when rapid proliferation was still occurring (an approximate doubling time of 48 hours was maintained to this passage number) and gross morphology was not altered. PEC were used only below passage 8 when rapid proliferation was still occurring in endothelial growth medium and gross morphology was maintained. The extended culture of PEC was necessary to attain reproducibility of the angiogenesis assays performed, for all of the assays were completed using endothelial cells isolated from 3 patients. This was done to minimize the effect of biological diversity on the assays, which would complicate the experiments if PEC from new patients were used for every assay run.

Another limitation of these studies is the utilization of the Matrigel assay to assess angiogenesis. Some debate exists over whether the tubule-like structures which endothelial cells form when plated on Matrigel or GFR-Matrigel represent capillaries as these tubule-like structures may or may not posses a lumen (46). Additionally, the matrix that endothelial cells are embedded in has been shown to partly control

the mechanisms utilized by endothelial cells to form tubules (46). Multiple different models for *in vitro* tubule formation were tested before GFR-Matrigel was selected to use in the study. Other *in vitro* models tested did not have the reproducibility that was required to be able to compare multiple different preparations of FcCM or could not be carried out on the number of samples at one time that the experimental design required for proper comparison. Thus, if a suitable method with adequate reproducibility and scalability could be found our results with FcCM would be enhanced by testing with a second model.

An additional limitation of the fibrocyte-like cell IUGR study is the type of hypoxic chambers that were used. These chambers were not connected to a controlled oxygen workspace thus every time the door of the incubator was opened or any manipulations or medium changes needed to be performed the cells had to be removed from the incubator and were exposed to higher oxygen levels. Efforts were made to minimize the effect of the higher oxygen exposure when making conditioned medium by producing the conditioned medium overnight when the incubator doors were not opened for a continuous ~18hr stretch of time and by pre-incubating the medium for a minimum of 4 hours before addition to the cells. However while the cells were growing, exposure to higher oxygen tensions were likely experienced due to maintenance of cultures and other experiments. This environment is therefore not particularly ideal for studying prolonged hypoxia, but may mimic the fluctuating nature of oxygen delivery to the placenta *in vivo*.

As highlighted in the recent article by Chen et al. (2013), the actual pericellular concentration of oxygen seen by cells in culture is another important point to consider (47). Chen et al. showed that the pericellular oxygen concentration of cultured primary trophoblasts under standard culture conditions (20-21% O_2 5% CO_2) is <0.6% O_2 (47). This value is even lower than has been previously observed with other cell types and illuminates the point that the atmosphere measured in the incubator is unlikely to match what is seen at the cell surface (47,48). The pericellular oxygen concentration in cell culture is determined by the balance of diffusion of oxygen through the culture medium and consumption of the oxygen by the cells. Therefore, due to the varying metabolic rates of different cell types and the variable diffusional capacity of oxygen in different culture medium the actual pericellular oxygen concentration seen by cells undoubtedly varies widely. This is, therefore, a general limitation of cell culture that must be considered when working with hypoxic exposure. Future studies examining chronic hypoxia in placental cells thus would benefit from close monitoring of pericellular oxygen concentrations so the actual oxygen concentration observed by the cells can be determined and properly replicated by others and adjusted to represent more physiologic concentrations if possible.

6.10 Future Directions in Placental Growth- Development of New Models

When all of the results in this thesis and the future directions already presented are considered together it becomes clear that there is a need to develop new models to examine placental growth. Trophoblast differentiation and placental angiogenesis are potential targets for therapeutic treatment of IUGR and sophisticated models to examine the individual processes and the potential cross-talk between these processes are required. Co-culture models allowing close association of trophoblasts and endothelial cells to mimic the environment of the vasculosyncytial membrane have yet to be published and would constitute an important model for future research. Additionally, a study examining whether angiogenesis could be examined with current explant models is required. As already discussed in section 6.3 there are many different directions in which trophoblast differentiation models could be further developed to solve issues surrounding the current commonly used models.

Finally, the field would also greatly benefit by increasing usage of the mouse placenta to examine both angiogenesis and trophoblast differentiation in addition to the *in vitro* models currently used. Development of tissue-specific knockout animals or making-use of/thoroughly investigating the placental phenotype of already developed knockout strains would help the field to more effectively tap into the huge amount of knowledge that could be discovered with this powerful tool. Utilizing this technology effectively also necessitates intensive research into the

cellular constituents and timing of key developmental processes within the murine placenta so that proper parallels to human placental development and structure can be drawn. Increased investigation of the mouse placenta and its similarities and differences to the human placenta with regards to key regulatory processes would also be advantageous when it comes to testing potential therapeutic treatments targeting placental growth. Meaningful examination of how therapeutic treatment may affect the placenta, and whether similar pathways are likely to be affected in humans will enhance the chance of successful drug trials and hopefully lead to the production of safe and efficacious therapies.

6.11 Conclusions

In conclusion, the studies presented in this thesis provide valuable information for understanding mechanisms of placental growth with regards to the processes of trophoblast differentiation and placental angiogenesis. These results suggest four important conclusions: 1) trophoblast differentiation is an apoptosis-independent event; 2) usage of the differentiation agent forskolin in future studies examining trophoblast differentiation must consider the multiple effects of forskolin on trophoblastic cells; 3) the placental stroma contains a population of fibroblastic cells capable of supporting angiogenesis; and 4) inherent differences exist in the production of angiogenic factors by placental cells from pregnancies complicated by IUGR but these differences cannot be attributed to exposure to a chronically hypoxic environment *in vitro*.

6.12 References

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Chapter 7: Appendices

7.1 Appendix A: Pilot Data for Fibrocyte-like cell conditioned medium loses its ability to stimulate angiogenesis in a model of chronic hypoxia

Initial studies were carried out as pilot data to examine whether chronic hypoxia would have a reduced ability to stimulate PEC angiogenesis *in vitro*. In this study fibrocyte-like cells isolated from normal placentas (n=5) were maintained in an environment of 6 or 8% O_2 5% CO_2 (physiologic normoxia) or 3% O_2 5% CO_2 (hypoxia) chronically as detailed in section 4.3.2 and conditioned medium was produced and collected as explained in section 5.3.4. Placental endothelial cells (PEC) were also maintained in an atmosphere of 8% O_2 5% CO_2 or 3% O_2 5%CO_2 chronically (a minimum of one passage or ~1week) as outlined in section 4.3.3. Angiogenesis assays were carried out using PEC that were isolated from the same placenta and half of the cells at the time of thawing were plated in normoxia and the other half in hypoxia. Thus the assay was carried out using cells from the same individual, at the same passage but the cells had been maintained at different oxygen tensions. Angiogenesis assays were carried out as outlined in section 4.3.7 on GFR-Matrigel with the assay run at 8% O_2 if the PEC had been maintained in that environment with FcCM also produced at 8% O_2 or if the PEC had been maintained at 3% O_2 then the assay was run at 3% O_2 with FcCM produced at 3% O_2. FcCM was examined for the relative abundance of angiogenic factors utilizing ELISA as detailed

in section 4.3.9. with FcCM that had been produced at either 6% O_2 5% CO_2 or 3% O_2 5% CO_2.

Maintenance of PEC in chronic hypoxia did not result in any alterations in the area covered by tubule like-structures for either the positive (EGM-2MV) or negative (EBM) controls (Figure A-1a and b) therefore we concluded that the ability of PEC to form tubule-like structures was not altered by the environment in which they were maintained and the assay was run at. There was a trend towards decreased formation of tubule-like structures ($p=0.07$) when FcCM produced in chronic hypoxia was utilized (Figure A-1c). No statistically significant differences were found in the abundance of any angiogenic growth factors between FcCM produced at normoxia (6% O_2) or hypoxia (3% O_2).

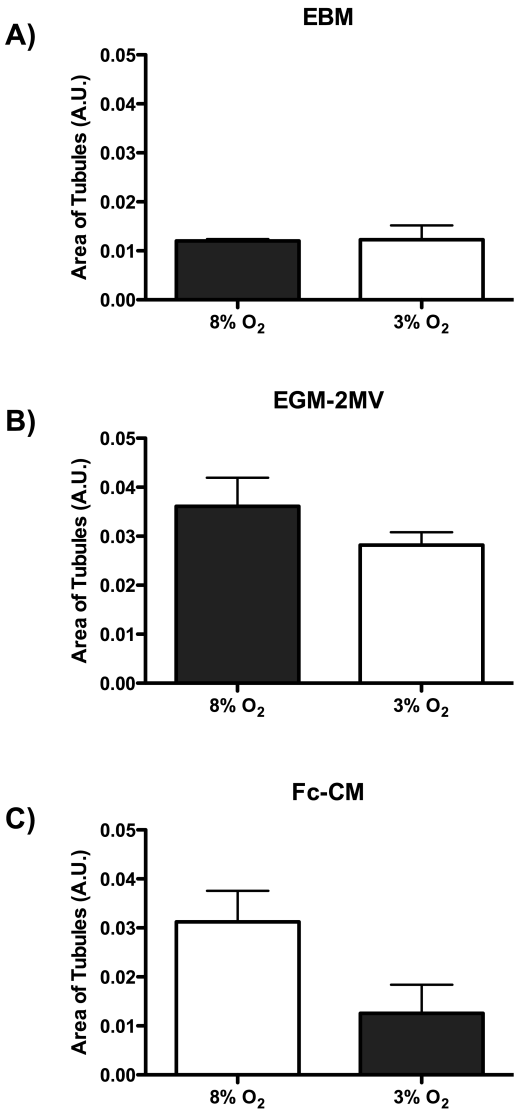

Figure 7-1: Pilot data examining the ability of PEC to form tubule-like structures after maintenance in chronic hypoxia

A) Summary graph of the area covered by tubule like structures after treatment with negative control EBM at physiologic normoxia (8% O_2) and hypoxia (3% O_2); mean +/- SEM; n=5; students t-test; p=0.94; B) Summary graph of the area covered by tubule-like structures after treatment with positive control EGM-2MV at physiologic normoxia (8% O_2) and hypoxia (3% O_2); p= 0.30; C) Summary graph of the area covered by tubule-like structures after treatment with FcCM that was produced in an atmosphere of 8% O_2 or 3% O_2 with the assay run at either physiologic normoxia (8% O_2) and hypoxia (3% O_2); p=0.07.

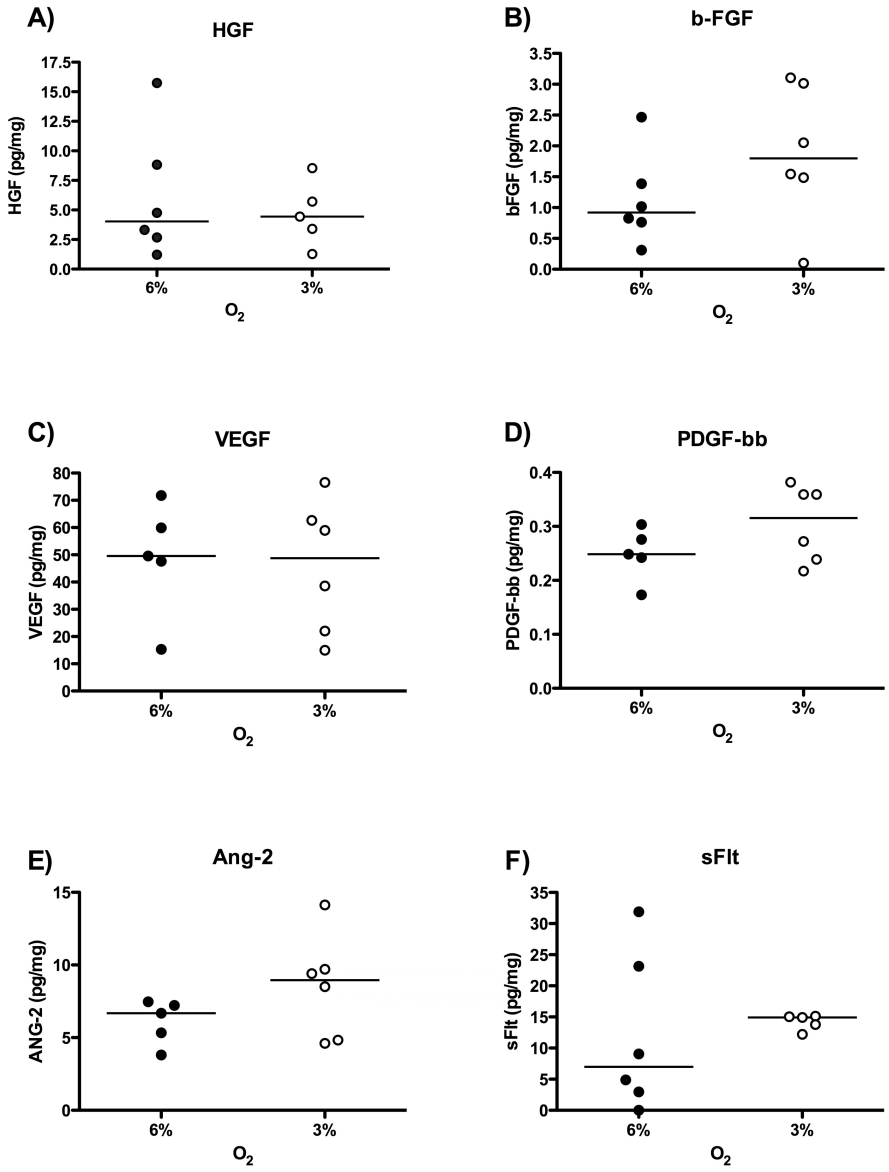

Figure 7-2: Abundance of angiogenic factors in FcCM produced in normoxia and chronic hypoxia

A) Pro-angiogenic HGF; line represents median; n=5-6; students t-test. B) Pro-angiogenic b-FGF; C) Pro-angiogenic VEGF; D) Pro-angiogenic PDGF-bb; E) Pro-angiogenic Ang-2; F) Anti-angiogenic sFlt.

7.2 Appendix B: Angiogenic Proteome Assay Results

See section 5.3.8 for methods.

Table 7-1: Factors Identified in FcCM by the Angiogenic Proteome Assay
IUGR= FcCM from fibrocyte-like cells from IUGR pregnancies; 6% and 1%= O_2; *Italicized factors* = anti-angiogenic; ND= not detected.

Angiogenic factor	Normal (6%)	Normal (1%)	IUGR (6%)	IUGR (1%)
Activin-A	0.2314977	0.206626	0.4232653	0.1028459
Angiogenin	0.4216366	0.501718	0.3381295	0.560820
Angiopoietin-1	0.6824245	0.372132	1.245365	0.3804134
Angiopoietin-2	ND	0.080294	0.1758468	0.1464094
Angiostatin	ND	0.042340	0.1175854	0.1102937
Amphiregulin	ND	0.004016	0.06209488	0.09357259
Artemin	ND	0.013836	0.06972478	0.1032114
Coagulation Factor III	0.2378643	0.482647	0.3209052	0.3711028
CXCL16	ND	0.061477	0.05801911	0.04128373
DPPIV	ND	0.014297	0.08110687	0.05177052
EG-VEGF	0.2986754	0.251367	0.3596807	0.1734933
Endostatin	ND	0.062371	0.08252852	0.0467208
Endothelin-1	ND	0.149931	0.2530671	0.2811685
FGF-acidic	0.06274173	0.122485	0.0957761	0.09162313
FGF-7	ND	0.011260	0.04536213	0.2169928
GDNF	ND	0.109609	0.1102685	0.09485862
GM-CSF	ND	0.091022	0.04628515	ND
HB-EGF	ND	0.069760	0.118222	0.07660921
HGF	0.1648388	0.118140	0.1909722	0.2829856
IGFBP-1	0.09836154	0.137985	0.3225549	0.1696282
IGFBP-2	0.004651251	0.125959	0.2684277	0.1836294
IGFBP-3	ND	0.994416	0.6884155	0.134993
IL-8	1.335559	1.169212	0.6359752	0.04330502
IL-1b	ND	ND	ND	0.049683

Table 7-1 cont.:

Angiogenic factor	Normal (6%)	Normal (1%)	IUGR (6%)	IUGR (1%)
TGF-b1	ND	0.070447	0.04032622	0.05097749
MCP-1	ND	ND	0.06981497	0.04055268
MIP-1a	ND	0.120463	0.1537316	0.07935028
MMP-8	ND	0.213929	0.1411516	0.1002796
MMP-9	0.271844	0.372688	0.4993424	0.4580058
PTX-3	0.0702965	0.267459	0.1888274	0.1105613
PD-ECGF	ND	0.124503	0.1696456	0.08964691
PDGF-aa	ND	0.108474	0.0762248	0.03073073
Persephin	0.2739407	0.148001	0.2143906	0.1056497
PF4	0.373800	0.281088	0.3209901	0.2231383
PlGF	ND	0.045405	0.09821625	0.08000949
Prolactin	ND	ND	0.1038145	0.06213476
Serpin B5	ND	0.128076	0.1413128	ND
Serpin F1	0.8640858	0.603558	1.240253	0.7918224
TIMP-4	ND	0.183173	0.1946643	0.1104332
TSP-1	1.739916	1.163915	1.345277	1.210771
TSP-2	ND	0.101265	0.07997874	0.03854547
uPA	0.5199562	0.173902	0.7463923	0.6238154
VEGF	0.5540196	0.777874	0.5959531	0.5071761