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Cytotrophoblast Differentiation in the First Trimester of Human Pregnancy

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The University of Auckland
New Zealand
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Dedications

I would like to dedicate this research to all my friends who have supported me. They made me laugh when no progress was being made, they had faith in me when they couldn’t understand what I was on about, they pretended to be interested when they didn’t know what a trophoblast was, and they learnt to stop asking when I would be finished. I would particularly like to dedicate this work to the following people for their unlimited love, personal support and charity: Cam Owens, Glenn Whyte, Katherine East, Kevin Webb, Joff Morpeth, Josh Wilson, Richard Carter, Sarah Fleming and Nicholas Smeeton. Finally, I would like to dedicate this work to my family for their ongoing support and faith in me, and in particular for their financial assistance without which finishing this research would not have been possible.
Acknowledgements

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Abstract

In the first trimester of human pregnancy specialised placental cells, termed cytotrophoblasts differentiate into extravillous trophoblasts (EVTs), which grow out from the placenta and invade into the maternal decidua, acting to physically attach the placenta to the decidua, and adapt the uterine spiral arteries to support pregnancy. A proportion of these EVTls also temporarily occlude the spiral arteries for approximately the first 10 weeks of gestation, preventing maternal blood flow to the placenta and creating a low oxygen environment in which placental and fetal development occur. The processes of trophoblast outgrowth and invasion, and the establishment of a low oxygen environment, are essential for the success of pregnancy, and inadequate trophoblast invasion into the uterus has been associated with recurrent miscarriage, pre-eclampsia and fetal growth restriction. However, the exact mechanisms that control the differentiation of cytotrophoblasts down the extravillous trophoblast lineage are poorly understood. Since the extent of this invasive process is unique to human implantation, animal models are of limited value in studying trophoblast invasion. Existing in vitro models have major limitations in that many are very difficult to quantify while others many not study the correct trophoblast population. The research in this thesis has focussed on the development of novel models by which to study cytotrophoblast differentiation, and the use of these models to further understand cytotrophoblast differentiation down the EVT lineage, and the regulation of EVT outgrowth by oxygen.

Methods

Outgrowth from first trimester villous explants was characterized using immunohistochemistry. Explant viability was investigated using dual staining with chloromethylfluorescin diacetate (CMFDA) and ethidium bromide, by examining DNA laddering, and by immunostaining sectioned explants over 96 hours of culture. The extended viability of cytotrophoblasts in multilayered cell islands in villous tips was exploited to isolate these cells using sequential trypsin digests of cultured villous explants. The trophoblast population obtained were characterized by immunohistochemistry. Finally, villous explants were cultured in either 1.5% or 8% oxygen and the frequency and area of outgrowths was quantified in order to determine the effect of gestation and oxygen on EVT outgrowth.
Results

Approximately 1/4 of explants cultured in 20% oxygen produced EVT outgrowth. Outgrowth formation and expansion resulted from proliferation of cells in the tips of anchoring villi, and EVTs within the outgrowth did not proliferate. The percentage of explants producing outgrowth declined as gestation increased from 8 to 12 weeks. Dual staining with CMFDA and ethidium bromide revealed degeneration of the syncytiotrophoblast by non-apoptotic mechanisms within 4 hours of culture, but this syncytiotrophoblast layer was able to be regenerated. The majority of cytotrophoblasts died within one week of culture, but despite this explants were able to produce EVT outgrowth for up to 3 weeks due to the extended survival of a specific set of cytotrophoblasts located in cell islands in the tips of anchoring villi. These surviving cells were able to differentiate into EVTs, but not regenerate the surrounding syncytiotrophoblast in the villus tip.

Trypsinization of first trimester villi after extended explant culture resulted in the isolation of a viable population of ‘putative EVT progenitors’ that did not syncytialise in culture, but were able to proliferate. 20% of these cells differentiated down the EVT lineage within 96 hours of culture. The putative EVT progenitors expressed markers previously localised to cytotrophoblasts in cell islands of anchoring villi, including αvβ6 integrin and FGFR-2. The addition of exogenous FGF-4 did not affect the differentiation of these cells into EVTs, nor did FGF-4 alter the frequency of EVT outgrowth from explants.

Culture in 1.5% oxygen significantly reduced the frequency and area of outgrowths in comparison to 8% oxygen. HLA-G and α1 integrin were both expressed throughout outgrowths with no difference in expression of these proteins between oxygen concentrations. Gestation influenced the response of explants to oxygen, with a significant differential response to oxygen concentration in placentae under 11 weeks of gestation but no differential response in placentae of 11 or 12 weeks.

Conclusions

In the first trimester, oxygen and gestational age regulate extravillous trophoblast outgrowth in both an independent and interdependent manner.
The cytotrophoblast population in the first trimester does not consist of one homogenous bipotent population. Rather there are at least two separate populations: 1) EVT progenitors that exist in the tips of potential anchoring villi that are likely to be committed to EVT differentiation and 2) monolayer villous cytotrophoblasts which are likely to be committed to syncytiotrophoblast differentiation. The second population is easily isolated by traditional enzymatic digestion methods whereas the first much smaller population can be isolated by exploiting their prolonged survival in explant culture.
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<tbody>
<tr>
<td>bHLH</td>
<td>basic helix look helix</td>
</tr>
<tr>
<td>CMFDA</td>
<td>chloromethylfluorescin diacetate</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor-1</td>
</tr>
<tr>
<td>CVS</td>
<td>chorionic villous sampling</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EVT</td>
<td>extravillous trophoblast</td>
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<tr>
<td>FGF-4</td>
<td>fibroblast growth factor-4</td>
</tr>
<tr>
<td>FGFR-2</td>
<td>fibroblast growth factor receptor-2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage – colony stimulating factor</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<td>HIF</td>
<td>hypoxia inducible factor</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>IGF</td>
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<td>IGFBP</td>
<td>insulin growth factor binding protein</td>
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<td>IVS</td>
<td>intervillous space</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>matrix metalloproteinase</td>
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<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
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<td>phosphate buffered saline solution</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
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<tr>
<td>PI GF</td>
<td>placental growth factor</td>
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<td>PS</td>
<td>phosphatidyl serine</td>
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<td>von Hippel-Lindau tumour suppressor protein</td>
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<td>transforming growth factor</td>
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<tr>
<td>TGFR</td>
<td>transforming growth factor receptor</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
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<td>VEGF</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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Chapter One:

Introduction
1.1 Placental development in the first trimester of human pregnancy

The human placenta is a fetal organ that is essential for the development of the embryo and the success of pregnancy. The placenta acts as an interface between the mother and developing fetus, playing important roles in implantation and nutrient and gas transport, along with immune, endocrine and metabolic functions (Boyd & Hamilton, 1970).

Placentation is initiated when the blastocyst makes contact with the epithelial lining of the uterus, leading to a series of events known as implantation. After fertilization in the oviduct, a series of symmetrical cell divisions results in a mass of totipotent cells termed the morula. The differentiation of the totipotent morulla cells is determined by their position, with the cells that lie on the outside of the morulla becoming the trophoectoderm, from which the placenta will form, and the inner cells forming the inner cell mass from which the embryo will be derived (Boyd & Hamilton, 1970).

The uterine endometrium is also transformed in preparation for implantation in a process called decidualisation. In humans, decidualisation involves rapid proliferation of the epithelial and stromal cells of the endometrium, followed by increased activity of the secretory glands and finally stromal cell differentiation whereby the cells surrounding the spiral arteries and beneath the uterine epithelium become plump and glycogen rich (Loke & King, 1996). The decidualised endometrium (decidua) acts as a physical matrix for placental anchorage, and the decidual extracellular matrix (ECM) undergoes rapid remodeling, with a reduction in collagen fibrils and type VI collagen, and an increase in type IV collagen, laminins 2 and 4 and heparin sulphate proteoglycan (Aplin et al., 1988; Mylona et al., 1995). In humans, spontaneous decidualisation is initiated in the normal menstrual cycle and will occur even in the absence of pregnancy (Loke & King, 1996). This trait sets humans apart from many other species such as the mouse in which the decidual reaction is triggered by implantation (Loke & King, 1996).

1.1.1 Implantation

The human blastocyst embeds itself between the mucosal cells of the decidualised endometrium on the 6th-7th day after fertilisation, at which point the placenta begins to form from the trophoectoderm (Loke & King, 1996). By the 8th day after ovulation the trophoectoderm has differentiated into an outer, multinucleated primitive syncytium (which will mature to form the syncytiotrophoblast) and an inner layer of primitive
mononuclear cytotrophoblasts. At this point fluid filled spaces appear between invaginations of the trophoectoderm termed the trophoblastic lacunae, which may be confluent with maternal vessels at the periphery of the implantation site (Enders, 1989). Villus development begins with the appearance of buds of cytotrophoblasts which lengthen and protrude into the primitive syncytium in the second week of gestation forming ‘primary villi’ (Loke & King, 1996). These buds are invaded by mesenchymal cells, converting them into ‘secondary villi’ (Boyd & Hamilton, 1970). Finally, via proliferation of the mesenchyme, these villi develop into ‘tertiary villi’, which contain fetal blood vessels and occasional macrophages (Hofbauer cells) in a core of mesenchymal connective tissue. The fetal and maternal circulations are separated by trophoblast at all times, ensuring that the fetal and maternal circulations never come into direct contact (Boyd & Hamilton, 1970).

1.1.2 Trophoblast differentiation

The mesenchymal core of placental villi is surrounded by a two layers of trophoblast from the third week of gestation – a layer of mononuclear cytotrophoblast stem cells, and an overlying continuous layer of multinucleated syncytiotrophoblast (Figure 1.1) (Boyd & Hamilton, 1970; Loke & King, 1996). The inner layer consists of mononuclear cytotrophoblasts which are commonly believed to be bipotent progenitors capable of either differentiating into syncytiotrophoblast or extravillous trophoblasts (EVTs). In the syncytiotrophoblast differentiation pathway, cytotrophoblasts fuse into the overlying non-proliferative, multinucleated syncytiotrophoblast, which forms a cell layer that covers the entire surface of the villous placenta, and provides an impermeable barrier to maternal blood. Thus, cytotrophoblast fusion into the syncytiotrophoblast supports its expansion and function. The syncytiotrophoblast plays an important role in human pregnancy, as it is the site of nutrient exchange and steroid and hormone synthesis required for normal fetal growth and development.
Cytotrophoblasts which differentiate down the EVT pathway are believed to arise from multilayered pools of villous cytotrophoblasts, sometimes referred to as ‘cell islands’, in the tips of anchoring villi (those villi that physically attach the placenta to the decidua). Cytotrophoblasts at the tips of anchoring villi break through the syncytial layer and expand outwards, forming ‘EVT outgrowths’ (Bischof et al., 2000; Boyd & Hamilton, 1970). EVT outgrowths from neighboring villi fuse to form an EVT layer, known as the cytotrophoblast shell, around the entire conceptus (Boyd & Hamilton, 1970). EVT’s from the cytotrophoblast shell invade further into the decidua in a process that is tightly regulated both temporally and spatially, and is essential for the success of pregnancy. However, although trophoblast differentiation is known to be regulated by a number of interacting factors, the exact mechanisms which stimulate cytotrophoblast differentiation down either the syncytiotrophoblast or EVT pathway, and the differences between cytotrophoblasts found in cell islands and those which fuse to form syncytiotrophoblast remain unclear.
1.1.3 Syncytiotrophoblast turnover

The multinucleated syncytiotrophoblast is continuously turned over throughout pregnancy, with an equilibrium existing between the continued fusion of underlying cytotrophoblasts into the syncytiotrophoblast layer, and the extrusion of aged nuclei. Structures known as ‘syncytial knots’, which consist of groups of aggregated nuclei with characteristic apoptotic features can be observed bulging out from the syncytiotrophoblast surface (Huppertz et al., 1998; Mayhew et al., 1999). These syncytial knots are shed from the syncytiotrophoblast and deported into the maternal circulation as a normal part of the process of syncytiotrophoblast turnover (Douglas et al., 1959; Wagner, 1968).

1.1.4 Villous branching and the intervillous space

As the human blastocyst invades deeply into the decidua during implantation, it is surrounded on three sides by the decidua capsularis, and decidua peritalis while deep to the invading trophoblasts lies the decidua basalis (Figure 1.2). As villous development occurs, changes also occur in the adjacent endometrium in what is known as the decidual reaction. Portions of the uterine glands adjacent to the trophoblast are eroded, and the maternal sinusoids are dilated and fill with plasma (Boyd & Hamilton, 1970). At this point the developing intervillous space is able to communicate with the sinusoids by channels through the cytotrophoblast shell (Boyd & Hamilton, 1970).

By the 4th week of gestation the placenta consists of villi of varying shape and length, depending on their position on the surface of the chorionic sac, with those villi in contact with the decidua basalis appearing generally longer and better vascularised than those in the region of the decidua capsularis (Boyd & Hamilton, 1970). By this stage the intervillous space (IVS) now consists of a complicated system of intercommunicating spaces, and has free communication with venous sinusoids at its periphery, however communications with the terminal branches of the spiral arteries have not yet been established i.e. maternal blood remains excluded from the IVS (Boyd & Hamilton, 1970).

Placental villi undergo rapid extension and branching through the second and third months of pregnancy, forming the villous tree structure characteristic of the mature human placenta (Loke & King, 1996). Stem villi form the foundation of the villous tree from which intermediate villi branch off. Terminal villi may arise from the lateral aspects
of stem villi, however the majority arise from ‘syncytial sprouts’ (surface protrusions of the villi as classified by Hamilton & Boyd, 1966) which cover the intermediate villi (Burton, 1987; Hamilton & Boyd, 1966). Terminal villi therefore form the outer tips of the villous placenta from which EVT outgrowths may arise. In the third month of gestation the villi regress from the decidua capsularis region of the chorionic sac and eventually define the placental margin overlying the decidua basalis (Boyd & Hamilton, 1970).

Figure 1.2 – Development of the placenta and fetal membranes. a) At 10 weeks of gestation the embryo is surrounded by the chorion frondosum (CF), which is covered by the capsular decidua (CD) that is continuous with the basal decidua (BD). b) At 12 weeks of gestation the original chion frondosum has differentiated into a thick placenta (P) and the membranes that surround the inner amniotic cavity (AC) which consist of the inner amnion (A), intermediate chion leave (L) and capsular decidua (CD). c) From 17 weeks of gestation the membranes come into close contact with the uterine wall, and the capsular decidua fuses with the parietal decidua. From Benirshke & Kaufmann, 2000.
1.1.5 Trophoblast remodeling of the spiral arteries

Where the cytotrophoblast shell lies over the maternal spiral arteries a subset of EVTs termed endovascular trophoblasts migrate down these arteries and interdigitate between and replace the arterial endothelial cells by mimicking the endothelial cell adhesion molecule repertoire (Zhou et al., 1997). Furthermore, these trophoblasts form plugs which occlude the vessel lumen, preventing maternal blood flow into the IVS before the end of the first trimester (Hustin & Schaaps, 1987). Therefore placental development, and the initial growth and organogenesis of the fetus, occur under hypoxic conditions (approximately 3% O2) (Rodesch et al., 1992). The concept of placental development in these, physiologically normal, low oxygen conditions will be addressed in more detail in section 1.4. A further trophoblast population, termed interstitial trophoblasts, invade between the stromal cells of the decidua and appear to preferentially migrate towards and encircle the uterine spiral arteries where they are believed to prime the arteries for endovascular trophoblast invasion (Kam et al., 1999). This combination of events produces the “physiological changes of pregnancy”, resulting in a loss of smooth muscle and the elastic and collagenous ECM from the spiral arteries, and its replacement with a fibrin-based deposit (fibrinoid) (Robertson et al., 1967). The remodeling of the spiral artery walls decreases the resistance of the spiral arteries to maternal blood flow, enhancing the supply of maternal nutrients to the growing fetus and making the uteroplacental blood supply independent of maternal vasoconstriction (Benirschke & Kaufmann, 2000). Therefore, by the end of the first trimester when the trophoblast plugs dislodge, the remodeled spiral arteries are large flaccid, non-vasoactive tubes that are able to accommodate the large increase in blood flow required by the developing fetus.

Invasion by endovascular and interstitial trophoblasts extends through the endometrium to reach the border of the decidua and myometrium by the 8th week of pregnancy (Loke & King, 1996). Endovascular trophoblast invasion continues until the middle of the second trimester by which time trophoblast invasion has reached the inner third of the myometrium (Pijnenborg et al., 1981). Endovascular trophoblast invasion is not homogenous throughout the placental bed, with the greatest number of arteries and depth of trophoblast invasion occurring in the central region of the placental bed (Brosens, 1988). The exact mechanisms that regulate endovascular trophoblast invasion are unknown, but the failure of endovascular trophoblasts to invade the myometrial
portion of the spiral arteries may result in fetal growth restriction or pre-eclampsia later in pregnancy (Brosens et al., 1972; Khong et al., 1986).

1.2 Methods used to study trophoblast differentiation in the first trimester
As human pregnancy is unique with respect to the extent of trophoblast invasion, common laboratory animal models are not often suitable to study human trophoblast differentiation, and studies of women in the early stages of pregnancy are limited to the use of non-invasive or diagnostic techniques for obvious ethical reasons (Cross et al., 2002; Enders & Carter, 2004). Therefore first trimester villous tissue, obtained from elective terminations of pregnancy, pregnancies that have ended in miscarriage, or chorionic villus sampling (CVS) are important resources for studying early human pregnancy. First trimester placenta can be used to study implantation and trophoblast differentiation in several different ways including isolating specific cell populations and growing small pieces of placenta termed explants in vitro. However, the nature of working with primary tissue means that these methods are usually time consuming and prone to variation, often requiring large numbers of replicates to produce meaningful results. Therefore, cell lines derived from choriocarcinomas or immortalized trophoblast populations are used by some researchers as a more efficient way to study trophoblasts in vitro.

1.2.1 Cell lines
The cell lines used to study trophoblasts can be broadly grouped into two categories, choriocarcinoma cell lines (e.g. Jeg3, Jars, BeWos) (Kohler et al., 1971; Pattillo & Gey, 1968), and those derived from immortalized isolated trophoblast populations (e.g. HTR-8/SVneo, TEV-1) (Feng et al., 2005; Graham et al., 1993). Choriocarcinoma derived cell lines have been used in many studies to examine the effects of multiple factors including cytokines, growth factors and oxygen conditions on trophoblast invasion, both by measuring the expression of enzymes that facilitate trophoblast invasion such as MMPs and urokinase plasminogen activator and their regulators, and by their ability of trophoblasts to invade into the ECM substrate Matrigel (Bischof et al., 2000; Hoang et al., 2001; Iwaki et al., 2004; Kilburn et al., 2000).

However, the use of cell lines to study trophoblast differentiation has several major drawbacks as these cells have significant differences from non-transformed trophoblasts
and therefore results derived from such studies may not accurately represent the nature of normal EVT differentiation or invasion in vivo. For example, by virtue of their immortalisation cell lines are proliferative, which is not a feature of differentiated EVTs in vivo. In addition, choriocarcinoma derived cell lines are characterized by their transformation from normal EVTs to cancerous cells which have a highly invasive phenotype. Therefore, using these immortalized cells to study the effects of culture conditions on cell proliferation or invasion may provide an inaccurate representation of EVTs in vivo. Studies using cell lines do serve a purpose in determining other aspects of placental biology, for example cytokine secretion where results can be compared to baseline levels, and provide a rapid way to run preliminary experiments. However, for the study of the specific process of trophoblast differentiation more relevant models of study are being increasingly used.

1.2.2 Isolated cytotrophoblasts

The isolation of trophoblasts from first trimester placentae is a common way to study trophoblast differentiation. This technique has the advantage over cell lines that it uses primary tissue, and therefore enables researchers to study the cells of interest directly. The most commonly isolated cell population is cytotrophoblasts. One of the most important factors in isolated cytotrophoblast populations is the purity of the population. In most common methodologies the cytotrophoblasts are contaminated with low levels of mesenchymal cells, in particular fibroblasts which multiply rapidly in culture (Frank et. al., 2001). Therefore, it is important to assess the purity of the cytotrophoblasts, usually with antibodies reactive with cytokeratin-7, a marker of trophoblasts, and/or antibodies reactive with vimentin, present in mesenchymal cells, to identify the proportions of each cell type (Frank et. al., 2001). In general, cultures containing greater than 95% trophoblasts are considered suitable for study. As cytotrophoblasts act as stem cells for both syncytiotrophoblast and EVTs they are fundamental to the study of differentiation down these pathways, and therefore isolation of these cells provides an important method to study this process. However, it is difficult to obtain large numbers of isolated cytotrophoblasts that either differentiate into an invasive EVT phenotype or proliferate following enzymatic digestion of first trimester placentae (Morrish et. al., 1997; Nagamatsu et. al., 2004a). Furthermore, while it is easy to measure the effect of culture conditions on cytotrophoblast phenotype and differentiation in monolayer culture, the
removal of these cells from their local microenvironment where the surrounding villous structure and paracrine signals may provide important cues to the direction of cell differentiation may result in an oversimplified understanding of cytotrophoblast differentiation in vivo.

1.2.3 Explant culture models

One of the most important features of an in vitro model being used to study trophoblast differentiation is its ability to mimic the situation in vivo as closely as possible. Explant culture models involve the culture of small sections of intact placental villi usually on an ECM component(s). Therefore, the use of explant cultures to study trophoblast differentiation is advantageous as the structure of the villi is maintained, with the retention of cell-cell communication and a microenvironment more akin to that which occurs in vivo. This is especially important in light of the complex interactions of the many paracrine and autocrine signals produced by the different cell types in the villus. These advantages have seen explant models being increasingly used by researchers studying placental function and trophoblast differentiation.

Explanting first trimester villi on substrates consisting of type I collagen or Matrigel results in the formation of EVT outgrowths from the tips of anchoring villi in contact with the ECM substrate (Aplin et al., 1999; Caniggia et al., 1997; Genbacev et al., 1992). EVT outgrowths in culture are able to be derived from two potential sources, firstly by the attachment and migration of cells in EVT columns already present at the villous tips, and secondly, by localized proliferation and differentiation of cytotrophoblasts in villous tips in contact with the ECM substrate. However, in any culture only a proportion of explants will produce viable trophoblast outgrowths, dependent on the culture conditions employed and the tissue gestation (Caniggia et al., 1997; James, 2001; Tartakover Matalon et al., 2005). The EVT outgrowths represent the first stages of villous cytotrophoblast differentiation into EVTs, however this system is unable to model further differentiation into endovascular trophoblasts or trophoblast interactions with the uterine cells. Recently, co-cultures of villous explants grown on sections of decidua peritalis from the same patient have been used to examine decidual blood vessel remodeling (Babawale et al., 1996; Dunk et al., 2003).
The thickness of the ECM used in culture models has the potential to affect trophoblast phenotype. Trophoblasts cultured on a slope of Matrigel varying in thickness from 1 to 60μm exhibit different properties depending on the underlying thickness of the Matrigel (Kliman & Feinberg, 1990). Flat aggregates of trophoblasts occur where the Matrigel is only 1-4μm thick, rounded aggregates with intercellular processes are present which rapidly degrade the Matrigel when it is between 4-14μm thick, and small aggregates of cells which show limited degradation are present at thicknesses greater than 14μm (Kliman & Feinberg, 1990). It is currently unclear how ECM thickness may modulate trophoblast behaviour, or the implications that this may have on trophoblast outgrowth and differentiation in vivo. However, it does illustrate the important role that the ECM plays in the regulation of trophoblast differentiation and behaviour, and this factor should be considered in the use of explant cultures.

To date the majority of villous explant models employ a three-dimensional system where the explant is cultured on a thick layer of Matrigel. In these systems the EVT outgrowths can expand and migrate in three dimensions by invading into the Matrigel (Aplin et al., 1999; Genbacev et al., 1992; Vivovac et al., 1995). However, it is also possible to culture villous explants on top of an extremely thin layer of Matrigel (James, 2001). This model has the advantage that EVT outgrowths are easily detectable and readily quantifiable growing in a two-dimensional formation across the Matrigel surface. In addition, growth factors often found in the Matrigel are kept to a minimum and are less likely to confound experimental results.

Villous explants are not without their disadvantages. Perhaps even more so than with other methods using primary placental tissue, inter-placental and particularly inter-explant results often show high levels of variance, requiring in a large number of replicates to obtain significant results (Newby et al., 2005). Furthermore, while beneficial for many experimental purposes, at times the maintenance of the tissue architecture can complicate the results obtained as the activities of individual cell types are unable to be identified. In this context, the viability of different cell types in the villus throughout the culture period can be an important consideration.
1.2.3.i The viability of first trimester villous explants in culture

During the course of explant cultures it is important to closely monitor the viability and tissue integrity of the explants, as a decline in the viability of specific villous cell populations may have a significant effect on the experimental outcome. However, the survival of all cell types in first trimester explants is not uniform. The syncytiotrophoblast has been reported to degrade rapidly in explant culture (Palmer et. al., 1997; Siman et. al., 2001; Watson et. al., 1995; Watson et. al., 1998). When examined by electron microscopy, the syncytiotrophoblast ultrastructure in fresh tissue showed condensed nuclei with an abundance of organelles, in particular mitochondria and a covering of microvilli (Watson et. al., 1998). However, after 4 hours culture in ambient oxygen, the syncytiotrophoblast was extensively vacuolated, no microvillous cover was present, organelles were difficult to identify, and mitochondria were swollen and irregular with extensive degeneration of the cristae (Watson et. al., 1998). This rapid degeneration of the syncytiotrophoblast was not observed in tissue cultured under hypoxic conditions, indicating that the syncytial degeneration seen under ambient oxygen conditions was a result of the exposure of the tissue to a physiologically superoxic environment (Watson et. al., 1998). This is likely to be due to the fact that the syncytiotrophoblast layer does not express the antioxidant enzyme superoxide dismutase that provides protection against oxygen free radicals during the first trimester (Watson et. al., 1997; Watson et. al., 1998).

The morphological degeneration of the syncytiotrophoblast in first trimester placental explants is supported by a range of biochemical evidence showing a decrease in cellular viability. For example, there is a decrease in the concentration of trophoblast derived hormones, such as hCG, in the culture medium, that are likely to arise from passive release as a result of degradation rather than active secretion, as it is accompanied by the release of the cytoplasmic enzyme lactate dehydrogenase, which indicates the breakdown of tissue integrity (Watson et. al., 1995). Furthermore, metabolism of labeled amino acids is decreased, indicating a decline in protein synthesis that is usually associated with a decline in cellular viability (Genbacev et. al., 1992). However, it is a feature of many studies that only broad biochemical markers treating the whole villus as one are used to provide a gauge of explant viability without accurately determining the viability of specific cell types relevant to the study (Kenis et. al., 2005; Polliotti et. al., 1995; Sooranna et. al., 1999; Watson et. al., 1995).
Recent evidence has emerged that despite the initial rapid degeneration of the syncytiotrophoblast, this layer is able to regenerate itself within 48 hours in culture as the underlying cytotrophoblast layer remains viable (Figure 1.3) (Palmer et al., 1997). Palmer and colleagues examined first trimester chorionic villi by electron microscopy over a period of 120 hours in culture. The syncytiotrophoblast showed clear degeneration within 24 hours, followed by the formation of a new syncytiotrophoblast layer derived from the viable underlying cytotrophoblasts which was largely formed by 48 hours in culture, and was maintained until at least 120 hours in culture (Palmer et al., 1997).

![Figure 1.3](image)

This rapid degeneration of the syncytiotrophoblast raises some concerns over the use of explant models in the study of syncytiotrophoblast function. In such studies researchers should be aware of the rapid degeneration of the syncytiotrophoblast and design experiments taking this into account. Researchers using explant models for other purposes should also be aware of the limited viability of the tissue in culture.

1.2.4 *In vitro* models as a method to study trophoblast differentiation

Trophoblast differentiation is not one discrete event that is able to be measured directly but rather a continuous and complex process involving multiple molecular changes in the cell regulated by a number of factors. Therefore, several different methods of measuring
trophoblast differentiation indirectly are used in the culture models described. Many methods of measuring trophoblast differentiation utilize proteins that are known to change as trophoblasts differentiate \textit{in vivo}. These proteins act as surrogate markers of the differentiation process and can be broadly grouped into several areas including cell proliferation (e.g. Ki67, PCNA and molecules involved in the regulation of the cell cycle such as p21 and p27), enzymes involved in invasion (e.g. matrix metalloproteases (MMPs) and urokinase plasminogen activator), adhesion molecules facilitating migration (e.g. integrins and cadherins) and proteins that are upregulated in differentiated trophoblasts to avoid immune attack (e.g. HLA-G and Fas). Invasion assays are also used to determine the depth that trophoblasts are able to invade through Matrigel as a marker of their invasive capabilities (Lacroix \textit{et. al.}, 2005; Pavan \textit{et. al.}, 2004; Zygmunt \textit{et. al.}, 1998b).

One limitation in the field of trophoblast biology is that the use of different models to study the same questions is often seen to produce conflicting results. Therefore, it is important to carefully consider the suitability of the model used to the study being undertaken, and in particular the whether the surrogate markers used to measure trophoblast differentiation accurately represent the process in the model used. For example, when measuring MMPs as a marker of trophoblast invasion it is necessary to look at the activity of MMPs, not just their expression, and also to examine MMP activity in the context of the relative activity of tissue inhibitors of matrix metalloproteases (TIMPs) that regulate MMP activity \textit{in vivo}. Furthermore, when examining the literature the points of difference between studies using different models that may affect the comparability and reproducibility of the results in other models must be taken into account. Finally, placentae ranging from 7 to 12 weeks of gestation are commonly studied, and it is important to be aware that, as the \textit{in vivo} environment changes significantly over this period, with a rapid increase in placental size, and the onset of maternal blood flow into the IVS, placentae at either end of this gestational range may not necessarily show the same experimental responses.

1.3 Trophoblast differentiation

Trophoblast differentiation is characterized by tightly regulated and specific molecular changes involving adhesion molecules, molecules that induce immunotolerance, cell cycle
regulators and proteases. However, the exact mechanisms which direct cytotrophoblast differentiation into either the syncytiotrophoblast or EVT lineages are not clearly understood. As EVTs move away from the villous placenta they progressively differentiate from a proliferative phenotype to an invasive phenotype. This differentiation is thought to be essential to allow the EVTs to invade and transform the maternal decidua and spiral arteries. It remains unclear whether the loss of proliferative activity as trophoblasts differentiate into an invasive phenotype is due to a terminal differentiation of EVTs along the invasive pathway, inhibition of proliferation by decidual and placental factors, or a combination of both mechanisms. Understanding the factors that control trophoblast differentiation and invasion is complicated by the large network of interacting regulatory factors derived from both the placenta and the decidua that may be involved in the regulation of this process. Increasing our knowledge of this network, and how it functions in normal pregnancy, will provide valuable insights into how aberrations in trophoblast differentiation may compromise the success of pregnancy.

1.3.1 Characteristics of cytotrophoblasts

Villous cytotrophoblasts in the first trimester of pregnancy are commonly believed to be a bipotent progenitor cell population, capable of either fusing to form the overlying syncytiotrophoblast, or differentiating into EVTs. In order to understand what is involved in the process of differentiation down either of these pathways it is important to first consider the basic characteristics of the villous cytotrophoblasts.

In the first trimester the cytotrophoblast layer has a high rate of proliferation as the placenta is growing rapidly (Chan et. al., 1999). The estimated percentage of proliferating cytotrophoblasts at any one time varies greatly from study to study with most estimates ranging from 7-23% (Chan et. al., 1999; Genbacev et. al., 2000a; Xue et. al., 2003). However, there are differences in proliferation between different areas of cytotrophoblasts in the villi, with a greater percentage of the multilayered cytotrophoblasts present in the tips of anchoring villi staining with the proliferation marker Ki67 than the monolayer of villous cytotrophoblasts underlying the majority of the syncytiotrophoblast (Nishimura et. al., 2004; Vivovac et. al., 1995). It is possible that
this difference in proliferation may be important for the different roles of cytotrophoblasts in different locations in the villus.

Trophoblast populations express characteristic adhesion molecules that allow them to control cell-cell and cell-ECM adhesion, and are important in facilitating trophoblast migration and interaction with the local microenvironment. Integrins are adhesion molecules formed by heterodimers composed of $\alpha$ and $\beta$ subunits which pair in differing combinations to form ECM receptors with distinct specificities, which show tightly regulated changes in expression as trophoblasts differentiate (Hemler et al., 1987; Hynes, 1987). Villous cytotrophoblasts express $\alpha_6\beta_4$, $\alpha_V\beta_5$ and $\alpha_V\beta_6$ integrins. $\alpha_V\beta_6$ integrin in particular is unique in that it is only expressed on cytotrophoblasts at sites of EVT column initiation, and may therefore act as a marker of, or play a role in, cytotrophoblast differentiation down the EVT pathway (Zhou et al., 1997). $\alpha_6\beta_4$ integrin is present at the basal cell surface where the cytotrophoblasts contact the villous basement membrane, in areas of contact between cytotrophoblasts and the syncytiotrophoblast and in some cells at the beginning of the EVT columns, and is believed to play a role in anchoring trophoblasts to the basement membrane (Aplin, 1993; Kemp et al., 2002). A further family of adhesion molecules expressed on cytotrophoblasts and involved in trophoblast differentiation are the cadherins. Cadherins are transmembrane glycoproteins that function as homotypic cell-cell adhesion molecules and are involved in cell-cell communication by establishing intercellular adherens junctions (Hirano et al., 1987). E-cadherin is strongly expressed by villous cytotrophoblasts on the cell surfaces that contact neighboring cytotrophoblasts and the overlying syncytiotrophoblast (Zhou et al., 1997).

1.3.2 Are villous cytotrophoblasts bipotent progenitors?

To date it has been assumed that cytotrophoblasts are bipotent progenitors that are able to differentiate into either syncytiotrophoblast or EVTs, however there is not a large body of direct evidence for this assumption. It is possible that there are in fact two subpopulations of villous cytotrophoblasts that may each be committed to different trophoblast lineage pathways. The development of EVT outgrowth from anchoring villi in explant cultures only occurs from the tips of villi, and not in other regions of the villous tree (Aplin et al., 1999). This suggests that different parts of the villous tree may
have different potential for development. It is possible that these regional differences in
developmental potential may arise from intrinsic cytotrophoblast differences, which may
in turn alter the paracrine signals involved in trophoblast differentiation. This raises the
possibility that, rather than one bipotent population, different sub-populations of
cytotrophoblasts may exist with different developmental potential.

1.3.3 Cytotrophoblast differentiation into syncytiotrophoblast

The syncytiotrophoblast is a unique multinucleated cell layer created by cytotrophoblast
fusion that forms the surface layer of the villous placenta. Cytotrophoblast fusion to
form the syncytiotrophoblast has been demonstrated by a number of methods including
microinjection of fluorescent dye into cytotrophoblasts, desmoplakin staining to outline
cytoplasmic membrane borders, and electron microscopy (Morrish et. al., 1998).
However, the physiological mechanisms behind this unique process are poorly
understood. Indeed, it is unclear whether fusion is driven by the cytotrophoblasts
themselves, or induced by the overlying syncytiotrophoblast. Older ultrastructural and
enzyme histochemical studies have suggested that ageing areas of the syncytiotrophoblast
were preferentially involved in fusion, indicating that fusion could be driven by areas of
the syncytiotrophoblast that require replenishment (Kaufmann, 1972; Kaufmann &
Stark, 1972; Martin & Spicer, 1973; Potgens et. al., 2004).

A number of molecular mechanisms have been described that control cytotrophoblast
differentiation and fusion to form the syncytiotrophoblast. Prior to fusion, villous
cytotrophoblasts have been reported to initiate the early stages of the apoptosis cascade
in order to facilitate fusion (Huppertz et. al., 1999). Upon fusion, the apoptotic cascade is
arrested until the syncytiotrophoblast is shed as part of the natural process of
syncytiotrophoblast turnover (Castellucci et. al., 1990; Huppertz et. al., 1999). One of the
key events for cytotrophoblast fusion, initiated early in the apoptotic cascade, is the
externalisation of phosphatidyl serine (PS), a negatively charged phospholipid normally
confined to the inner plasmalemmal leaflet (Adler et. al., 1995; Katsuragawa et. al., 1997).
The externalisation of PS may provide negative charges at the cell surface that are
required for the action of fusogenic proteins, such as syncytin (Blond et. al., 1999; Martin
& Ruysschaert, 1997; Mi et. al., 2000; Potgens et. al., 2002; Yu et. al., 2002).
In comparison to other trophoblast populations the syncytiotrophoblast has relatively low levels of adhesion molecule expression. As the syncytiotrophoblast constitutes the outer layer of the placenta, lower levels of adhesion molecule expression may allow individual villi to remain separate and fluid to circulate through the IVS and prevent leukocyte adhesion. The adhesion molecules expressed in the syncytiotrophoblast are similar to those in the cytotrophoblast from which it was derived and include α1, α5 and αv integrins (Kertesz et. al., 2000; Qin et. al., 2003).

Finally, syncytiotrophoblast formation is influenced by autocrine and paracrine signaling and can be induced by several factors in vitro, although it is unclear how each of these factors may stimulate syncytialization. Colony stimulating factor-1 (CSF-1), granulocyte macrophage-colony stimulating factor (GM-CSF) and epidermal growth factor (EGF) all increase the secretion of human chorionic gonadotrophin (hCG) and/or human placental lactogen (hPL) by the syncytiotrophoblast (Garcia-Lloret et. al., 1996; Morrish et. al., 1987). Syncytiotrophoblast production of hCG may act in an autocrine manner to further promote syncytialization (Shi et. al., 1993). The ECM components fibronectin and type I collagen also induce syncytialization in vitro (Morrish et. al., 1996). Therefore, in the study of trophoblast differentiation the culture conditions and presence of cytokines and ECM factors is an important consideration.

The number of factors influencing cytotrophoblast fusion into syncytiotrophoblast illustrate that this is a carefully controlled process which is important for the success of pregnancy. However, the large number of interacting mechanisms make it difficult to separate which factors direct trophoblast differentiation down this lineage and which factors are a consequence of this differentiation process. This is an ongoing problem in the study of trophoblast cell lineages and is made even more difficult by the even more complex process of cytotrophoblast differentiation down the EVT pathway.

1.3.4 Cytotrophoblast differentiation into EVT and the acquisition of an invasive EVT phenotype

The tightly regulated differentiation of multilayered cytotrophoblasts in the tips of anchoring villi into EVTs is essential for the success of pregnancy. EVTs are not a homogenous cell population and progressively differentiate from the proliferative...
phenotype found in the first few layers of the cell columns proximal to the villi into an invasive phenotype able to invade and interact with the maternal decidua. In order to invade the decidua successfully, invasive EVTs must upregulate the expression of enzymes able to facilitate local degradation of the ECM, alter their expression of adhesion molecules and evade maternal immune surveillance. The depth and extent of EVT invasion is tightly regulated by a number of factors including cytokines, decidual factors such as the ECM, and environmental factors such as oxygen concentration. However, the exact ways in which many of these regulatory factors act, and interact with each other, and how these factors may result in abnormal EVT invasion, remain unclear.

1.3.4.i Cell cycle arrest

In order for trophoblasts to proliferate they must enter the cell cycle. As cytotrophoblasts differentiate into EVTs and migrate away from the placenta they downregulate molecules associated with mitosis and upregulate cell cycle inhibitors, resulting in an exit from the cell cycle and a subsequent decline in proliferation (Genbacev et. al., 2000). EVTs proximal to the villi upregulate cyclin-dependant kinases (CDK) 2, cyclin B, and histone H3, indicating completion of the mitotic cycle (Genbacev et. al., 2000). Whereas, the EVTs in the distal portions of the columns upregulate cell cycle inhibitors such as p16, p27 and p57, while downregulating markers of commitment to the cell cycle such as CDK4 and 6, indicating complete withdraw of invasive EVTs from the cell cycle (Genbacev et. al., 2000).
Figure 1.4 A diagram of the cell cycle. The coloured cycling component shows the transition of cells through the first gap phase (G1), into the S phase in which cellular DNA is replicated, the second gap phase (G2), and finally mitosis (M phase) resulting in the production of two identical daughter cells. Daughter cells may then proceed through another replicative cycle, or leave this cycle to differentiate or enter the resting phase (G0).

1.3.4.ii Adhesion molecules and trophoblast interactions with the extracellular matrix

During decidualisation the ECM is remodeled, with a reduction in banded collagen fibrils and microfibrillar type VI collagen, and its replacement with fibronectin, type IV collagen, laminins 2 and 4 and heparan sulphate proteoglycan (Aplin et al., 1988; Mylona et al., 1995). As trophoblasts invade into the uterine decidua they interact not only with this maternally derived decidual ECM, but also with an extensive pericellular matrix secreted by the trophoblasts themselves (Damsky et al., 1994). The composition of the trophoblast derived ECM changes as the trophoblasts differentiate from villous to extravillous and finally invasive trophoblasts. Undifferentiated villous cytotrophoblasts produce laminin chains including merosin, but not fibronectin. However, by the time the trophoblasts enter the uterine decidua, the trophoblast-derived matrix includes oncofetal fibronectin, type IV collagen and $\alpha1$, $\beta1$, and $\gamma1$ laminin (Damsky et al., 1994; Korhonen & Virtanen, 1997). Therefore, the trophoblast adhesion molecule repertoire undergoes
specific changes with trophoblast differentiation which facilitate migration into and interaction with the remodeled decidua ECM.

As villous cytotrophoblasts differentiate into EVTs they undergo important changes in integrin expression, allowing interaction with a permissive ECM, which is important in stimulating the proliferation of EVTs proximal to the villus and differentiation along the EVT lineage (Aplin et al., 1999). As trophoblasts migrate away from the villous basement membrane the expression of α6β4 integrin, the ligand for the ECM component laminin, decreases thereby facilitating trophoblast migration, and is replaced by the expression of α5β1 integrin, the cell surface receptor for the extracellular matrix component fibronectin (Aplin, 1993; Aplin et al., 1999; Damsky et al., 1992). Fibronectin is abundant at the site of anchoring villus formation as it is present in the decidua and produced in its oncofetal isoform by EVTs in cell columns (Aplin et al., 1999). Expression of α5β1 integrin by EVTs is important for the maintenance of EVT outgrowth in vitro as treatment with antibodies to α5 integrin or fibronectin results in the rounding of cells and disruption of EVT outgrowth from first trimester villous explants (Aplin et al., 1999). As EVTs migrate out of cell columns and invade the uterine wall they continue to express the α5β1 integrin fibronectin receptor, but also upregulate α1β1 integrin, a collagen/laminin receptor (Damsky et al., 1992). Invasive trophoblasts interact with the decidua ECM primarily through the use of α1β1 and α5β1 integrins (Damsky et al., 1992).

E-cadherin expression has been reported throughout EVT cell columns, although a downregulation of E-cadherin expression by EVTs is observed as they differentiate and migrate down cell columns away from the villi (Floridon et al., 2000; Shih et al., 2002; Zhou et al., 1997). This decreased expression of E-cadherin is believed to contribute to the invasive capabilities of EVTs (Karmakar & Das, 2004). The adhesion molecules described above are often used as surrogate markers for different stages of trophoblast differentiation, and to establish the invasive capabilities of trophoblasts which are partly dependent on adhesion molecule interactions.
Figure 1.5 – Adhesion molecule expression in trophoblast differentiation.

The outgrowth of EVTs in columnar formation from explanted first trimester villi in vitro indicates that contact with a permissive ECM is sufficient to stimulate trophoblast outgrowth and differentiation (Aplin et. al., 1999). As trophoblast outgrowth will not occur on agarose, this process is not dependent on physical contact alone, indicating that the signaling process that stimulates EVT outgrowth involves interactions between a physiological ECM, such as Matrigel or type I collagen, and receptors on the villus such as integrins (Aplin et. al., 1999). Continued differentiation into an endovascular trophoblast phenotype does not occur in such villous explant models, as shown by the failure of trophoblasts in cell columns to upregulate adhesion molecules characteristic of an endovascular phenotype such as \( \alpha 4\beta 1 \) and \( \alpha V\beta 3 \) integrin (Aplin et. al., 1999).

The composition of the ECM that EVTs encounter has the ability to determine their invasive properties. Trophoblast interactions with laminin and type IV collagen,
mediated primarily through $\alpha_1\beta_1$ integrin, are critical for successful invasion \textit{in vitro}. Reducing trophoblast interaction with laminin and type IV collagen through the use of polyclonal antibodies blocks cytotrophoblast invasion by more than 80% (Damsky \textit{et al.}, 1994). In contrast, inhibition of associations with fibronectin by the use of anti-fibronectin antibodies does not block cytotrophoblast invasion \textit{in vitro}, calling into question the aforementioned role of the fibronectin receptor $\alpha_5\beta_1$ in EVT invasion (Damsky \textit{et al.}, 1994). Furthermore, it appears that individual ECM components may affect the expression of some integrins in EVTs, thereby determining the invasive properties of these cells, rather than a pre-determined integrin repertoire being responsible for ECM interactions (Kilburn \textit{et al.}, 2000). Kilburn and colleagues used a human first trimester cytotrophoblast cell line (HTR8-SVneo) to show that $\alpha_1$ integrin was upregulated, $\alpha_6$ integrin was downregulated, and $\alpha_5$ integrin expression was unaffected after transferring cells from culture on plastic or the individual ECM components of fibronectin, laminin and type IV collagen, to culture on Matrigel (Kilburn \textit{et al.}, 2000).

1.3.4.iii Extravillous trophoblast degradation of the extracellular matrix

An essential part of EVT differentiation is the ability of trophoblasts to degrade the ECM in order to facilitate invasion. EVTs accomplish this by several mechanisms including enzymatic degradation by matrix metalloproteases (MMPs) and cathespin L, the plasmin/plasminogen activator system, and physical processes such as phagocytosis.

The major group of enzymes involved in trophoblast degradation of the ECM are the MMPs. MMPs are zinc-dependent proteolytic enzymes that are able to cleave various constituents of the ECM. At least 21 MMPs have been identified (Tarrade \textit{et al.}, 2002). Most MMPs are secreted as inactive pro-enzymes that become activated by enzymes in the extracellular compartment (Bischof \textit{et al.}, 2000; Bischof \textit{et al.}, 2003). MMP activity is tightly regulated by four tissue inhibitors of metalloproteinases (TIMPs) which bind specifically to the highly conserved zinc-binding site of active MMPs (Fassina \textit{et al.}, 2000). Individual MMPs alone are not solely responsible for the invasive nature of EVTs, as MMP-9 knockout mice remain fertile, but rather form part of a network of interacting mechanisms that facilitate trophoblast invasion (Itoh \textit{et al.}, 1999).
The most prominent MMPs with respect to trophoblast invasion are the gelatinases MMP-2 and MMP-9, which have both been shown to mediate cytotrophoblast invasion into Matrigel \textit{in vitro} (Bischof \textit{et. al.}, 1995). MMP-2 is predominantly produced by the stromal cells of the villous mesenchyme and EVTs, whereas MMP-9 is produced predominantly by the villous cytotrophoblasts (Isaka \textit{et. al.}, 2003; Niu \textit{et. al.}, 2000). Trophoblast MMP expression changes with gestational age (Xu \textit{et. al.}, 2000). MMP-9 secretion by isolated human cytotrophoblasts increases more than 10 fold from when it is first detected at 7 weeks gestation (14.7ng/mL) to 11 weeks of gestation (167.7ng/mL) (Xu \textit{et. al.}, 2000). However, as the most important factor in MMP activity is the ratio between MMP and it’s inhibitor TIMP, this may be negated by a simultaneous 7 fold increase in TIMP-1 secretion from 6 to 11 weeks of gestation (Xu \textit{et. al.}, 2000). Conversely, MMP-2 production by isolated cytotrophoblasts declines between 6 (205.7ng/mL) and 11 weeks of gestation (32.3ng/mL) (Xu \textit{et. al.}, 2000).

MMP gene expression is primarily regulated at the transcriptional level. The promoter region of the inducible MMPs (MMP-1, 3, 7, 9, 10, 12, 13) is highly conserved and contains responsive elements that bind AP-1 transcription factors, c-ets oncogenes, a TGF\beta inhibitory element and the nuclear transcription factors Sp-1 and NF\kappaB (Bischof \textit{et. al.}, 2002). This transcriptional regulation of MMP mRNA is itself regulated by a large number of interacting hormones and cytokines, derived from the placenta, trophoblasts and the decidua. \textit{In vitro}, these factors include progesterone, hCG, EGF, gonadotrophin-releasing hormone (GnRH), leptin, tumour necrosis factor-\alpha (TNF\alpha), interleukin-1 (IL-1), IL-6, IL-10, leukemia inhibitory factor (LIF), and hepatocyte growth factor (Bischof \textit{et. al.}, 1995a; Bischof \textit{et. al.}, 2000; Bischof \textit{et. al.}, 2000a; Castellucci \textit{et. al.}, 2000; Chou \textit{et. al.}, 2003; Das \textit{et. al.}, 2002; Fukushima \textit{et. al.}, 2003; Licht \textit{et. al.}, 2001; Meisser \textit{et. al.}, 1999; Meisser \textit{et. al.}, 1999a; Nasu \textit{et. al.}, 2000; Roth & Fisher, 1999; Shimonovitz \textit{et. al.}, 1998). However, again care must be taken in interpreting the transcriptional regulation of MMP as expression alone is of no biological importance as a marker of trophoblast invasion unless changes in the expression of TIMPs are also considered.

The second major protease system facilitating trophoblast invasion is the urokinase-type plasminogen activator (uPA) system. uPA is secreted as an inactive pro-enzyme, which upon binding to its highly specific receptor is cleaved into an active molecule (Blasi, 1993). Trophoblast invasion is induced by the upregulation of the uPA receptor on the
cell surface of trophoblasts (Graham et al., 2000). Both free activated and receptor bound uPA are able to convert plasminogen into plasmin, a member of the serine proteinase family, which is then able to degrade several ECM components and activate growth factors and latent MMPs required for invasion (Mayer, 1990; Petersen et al., 1988; Saksela, 1985). However, there is no evidence to indicate that uPA increases trophoblast MMP activity (Canning et al., 2001). The uPA system is regulated by plasminogen activator inhibitors-1 and -2 (PAI-1 and PAI-2), which inhibit both free and bound uPA by forming irreversible covalent complexes (Fitzpatrick & Graham, 1998).

Figure 1.6 – Schematic diagram of the MMP and uPA systems. MMP and uPA transcription are regulated by a number of cytokines and hormones. uPA is secreted in a pro-enzyme form which is cleaved by binding to the uPA receptor. Active uPA is then able to convert plasminogen to plasmin. Plasmin is able to directly degrade the ECM, but also acts to convert pro-MMPs to their active form, resulting in further ECM degradation and allowing trophoblast invasion.

Finally, EVT utilizes phagocytosis to assist with the extensive tissue remodeling that occurs during trophoblast invasion of the decidua (Choy & Manyonda, 1998). In vitro, both primary EVTs and trophoblast cell lines created by DNA transfection were able to phagocytose sheep red blood cells, Staphylococcus aureus and bakers yeast cells, although less avidly than professional phagocytes (Choy & Manyonda, 1998). More importantly in the setting of trophoblast invasion, first trimester extravillous cell lines
have been shown to phagocytose collagen (Manyonda & Choy, 1999). Work from our laboratory has shown that in vitro, trophoblast derived cell lines can kill and phagocytose endothelial cells in a process which may parallel the invasion of the spiral arteries during implantation (Chen et. al., 2005). The combined mechanisms by which differentiated trophoblasts are able to degrade and invade the decidual ECM are essential for the success of pregnancy as an insufficient depth and extent of trophoblast invasion and transformation of the spiral arteries is associated with diseases of pregnancy including pre-eclampsia and intra-uterine growth restriction (Khong et. al., 1986; Robertson et. al., 1967).

In summary, the invasive mechanisms EVT utilize are essential for the success of pregnancy. However, the accuracy of in vitro models of trophoblast invasion are dependant on observations of the system as a whole, and inaccurate results will be obtained by researchers who look only at expression of proteolytic enzymes rather than the enzymatic activity of the system in its entirety.

1.3.4.iv Invasive trophoblasts evade immune surveillance

The developing placenta and fetus consist of genetic material from both the mother and father, and can therefore be considered a semi-allograft, and as EVTs differentiate and migrate away from the placenta they are directly exposed to the maternal uterine immune system. Therefore the question has been raised; why isn’t a maternal immune response generated to the placenta/fetus? One mechanism by which invasive EVTs avoid immune surveillance is by expressing several immunosuppressive proteins on their surface, allowing them to avoid attack by the maternal immune system. These immunosuppressive proteins are upregulated as EVTs differentiate into an invasive phenotype and migrate away from the placental villi, and therefore have been used in some instances as surrogate markers of trophoblast differentiation.

First, the absence of class I and II major histocompatibility complex (MHC) from villous trophoblasts protects these cells from the maternal immune T-cell mediated attack. EVTs also actively avoid immune attack by the expression of the class Ib HLA/MHC protein HLA-G, which can interact with receptors on cytotoxic T-lymphocytes and natural killer cells to inhibit the ability of these cells to induce cell lysis (Hofmeister &
Weiss, 2003). HLA-G expression is upregulated with trophoblast differentiation down the EVT lineage (McMaster et. al., 1995; Shorter et. al., 1993).

Secondly, Fas ligand is a protein present in both membrane bound and soluble forms that plays an important role in generating immune tolerance by binding to its receptor (Fas) on activated immune cells, resulting in apoptosis of the immune cell. Fas ligand expression allows invasive trophoblasts to evade immune surveillance by inducing apoptosis of Fas expressing T cells and CD56+ natural killer cells present at the materno-fetal interface (Guller & LaChapelle, 1999). Membrane associated Fas ligand expression has been reported in vivo in the syncytiotrophoblast and cytotrophoblasts, and is upregulated as EVT's differentiate into an invasive phenotype in vitro (Guller & LaChapelle, 1999; Kauma et. al., 1999; Runic et. al., 1996).

Finally, the third major factor involved in localized suppression of the maternal immune response is the presence of anti-inflammatory cytokines at the materno-fetal interface. A number of studies have suggested that there are alterations in the balance of Th1 and Th2 cytokines between normal pregnancies and pregnancies ending in recurrent miscarriage or pre-eclampsia, with elevated levels of inflammatory Th1 cytokines, and low levels of anti-inflammatory Th2 cytokines seen in women with recurrent miscarriage (Bowen et. al., 2002; Hennessy et. al., 1999; Lim et. al., 2000; Piccinni et. al., 1998; Raghupathy et. al., 1999; Raghupathy et. al., 2000). Indeed, isolated trophoblasts from normal pregnancies show a Th2 type cytokine production bias in vitro (Sacks et. al., 2001). In addition to their immunomodulatory function, cytokine expression at the maternofetal interface also influences the important processes of trophoblast differentiation and invasion. It was generally believed that inflammatory cytokines such as IL-1, IL-6 and IL-15 and TNFα, stimulate trophoblast invasion, whereas anti-inflammatory cytokines such as TGFβ, IL-10 and LIF inhibit trophoblast invasion and stimulate a proliferative trophoblast phenotype (Bischof et. al., 2002; Karmakar & Das, 2002; Librach et. al., 1994; Meisser et. al., 1999a; Todt et. al., 1996; Zygmunt et. al., 1998a; Zygmunt et. al., 1998b). However, it is now becoming apparent that such broad groupings of cytokines by their immunomodulatory functions may oversimplify their effects on trophoblast differentiation, and recent studies on individual cytokines including TNFα and LIF report opposing effects on trophoblast invasion than would be expected by their Th1/2 grouping (Bauer et. al., 2004; Poehlmann et. al., 2005). This demonstrates how the
control of the immune response to pregnancy is governed by a number of interacting factors and that the effects of molecules on cell differentiation and invasion should not be assumed as they are not always predictable.

1.3.5 Regulation of EVT differentiation from a proliferative to an invasive phenotype

A tightly regulated network of interacting signals from the placenta, decidua, and the ECM act to stimulate both the outgrowth of trophoblasts from placenta villi, and their progressive differentiation along the EVT lineage from proliferative but non-invasive trophoblasts proximal to the villi, towards a non-proliferative but invasive EVT phenotype (Aplin et. al., 2000). The full extent of this network remains to be determined but some of the more important factors that have been investigated and that are relevant to this thesis will be discussed in the following section.

1.3.5.i Transcriptional regulation of trophoblast differentiation

A range of transcription factors have been associated with trophoblast differentiation. Changes in transcription factor expression may occur not only to facilitate trophoblast differentiation, but also as a response to cellular changes arising from previous events in the differentiation pathway, or the activation of signaling pathways by autocrine and paracrine regulation from the surrounding environment. It has been difficult to separate these effects and therefore due to the complex number of interacting factors it is often only possible to associate changes in transcription factor expression with stages of differentiation rather than provide direct causative links.

Basic helix-loop-helix (bHLH) transcription factors have been shown to play important roles in murine trophoblast development and several human orthologues have now been identified (Knofler et. al., 2001). Hash-2 is expressed by EVTs of the placental bed in vivo and purified first trimester cytotrophoblasts in vitro where it is downregulated during differentiation into an invasive phenotype (Janatpour et. al., 1999; Jiang & Mendelson, 2003). bHLH transcription factors bind to their dimerization partners, either E-factors that activate transcription, or Id (Inhibitors of DNA binding) group proteins which inhibit transcription (Sun et. al., 1991). Id2 expression is downregulated as EVTs migrate
away from the villous and may contribute to their invasive phenotype (Janatpour et al., 2000).

The transcription factor GCMa (also referred to as GCM1) is downregulated during isolated cytotrophoblast differentiation to an invasive phenotype in vitro (Janatpour et al., 1999). GCMa is able to regulate the expression of the fusogenic protein syncytin and enhance syncytin-mediated fusion in the BeWo and Jeg3 choriocarcinoma cell lines (Yu et al., 2002). Furthermore, significant reductions in GCMa expression are observed in pre-eclamptic placentae, potentially contributing to the corresponding reduction in syncytin expression observed in pre-eclampsia (Chen et al., 2004; Lee et al., 2001). A further level of regulation may be provided by crosstalk between the GCMa and bHLH transcription factor families, as Id2 overexpression results in a 2.5 fold increase of GCM-1 mRNA (Janatpour et al., 1999).

Finally, the activator protein 1 (AP-1) family consists of dimeric transcription factors composed from the products of the Jun and Fos proto-oncogenes. Jun and Fos proto-oncogenes are expressed in vivo predominantly in EVTs, with some expression in villous cytotrophoblasts (Bamberger et al., 2004). AP-1 transcription factors have been associated with roles both in cytotrophoblast differentiation into syncytiotrophoblast, during which both c-Fos and JunB expression are increased in vitro, and in the differentiation of isolated cytotrophoblasts into an invasive phenotype, as AP-1 transcription factors are necessary, although not sufficient, for transactivation of the MMP-9 gene (Bischof et al., 2002; Dakour et al., 1999).

Whether the alterations in individual transcription factor expression are a cause or effect of trophoblast differentiation is not as important as the overall effects that these transcription factors have on the expression of placental and decidual factors that act on the trophoblasts to control this process.

1.3.5.ii Placental and decidual factors involved in the regulation of trophoblast differentiation

The human placenta regulates its own development by the production of specific cytokines and growth factors from the villous mesenchyme, cytotrophoblasts,
syncytiotrophoblast and EVTs which can act in an autocrine or paracrine manner to regulate trophoblast differentiation. This regulation of trophoblast differentiation is important because on one hand insufficient invasion can be detrimental to pregnancy, however uncontrolled invasion can also have the same effect, resulting in diseases such as choriocarcinoma (Fulop et al., 2004). As EVTs migrate away from the villi they are less influenced by the cytokines produced in the villous placenta, and exposed to increasing levels of cytokines produced in the decidua. The decidua plays an important role in regulating EVT differentiation and cytokines secreted by the decidua appear to have the overall effect of limiting trophoblast invasion (Bischof et al., 1998; Campbell et al., 2004). It is interesting that trophoblast differentiation is able to occur in tubal pregnancies in the absence of the decidua. Differentiated EVTs in tubal pregnancies exhibit a phenotypic switch from a proliferative to an invasive phenotype comparable to that of trophoblasts in uterine pregnancies (Goffin et al., 2003; Qin et al., 2003). However, trophoblast cell columns are markedly longer in tubal pregnancies than in intrauterine pregnancies, indicating that although the decidua is not necessary to trigger EVT invasion, it may act to limit the extent of EVT column formation (Goffin et al., 2003).

The effect of villous-derived regulatory factors on trophoblast differentiation can be demonstrated directly in explant models of EVT outgrowth. In vitro first trimester villous explant cultures have been reported to produce outgrowth in which the trophoblasts remain largely contiguous when the culture media is changed daily, however, leaving the culture medium on for several days without replacement results in large numbers of EVTs detaching from the outgrowth and migrating in a radial pattern away from the villi (Aplin et al., 2000). In our own laboratory we have also noted that in vitro trophoblast outgrowth is not sustained in the absence of the villus, but rather EVTs undergo cell death (James, 2001). By correlating the production or role of specific factors in explant models to the phenotype and appearance of the EVT outgrowth produced we are more accurately able to determine the role each factor plays in the complex regulation of trophoblast differentiation.

One important family of growth factors produced by the placenta are the insulin-like growth factors (IGFs), which are expressed in a wide range of tissues but are especially important in fetal and placental development where they play roles in regulating cell
proliferation, differentiation, migration and aggregation (Han & Carter, 2000). IGF-I is produced by the villous mesenchymal cells, and the addition of exogenous IGF-I stimulates EVT migration from villous explants in vitro (Aplin et al., 2000; Lacey et al., 2002). In vivo a concentration gradient is established where an IGF-I signal from the villous mesenchyme would be self-limiting as it diminishes with distance from the placenta. In addition to its effects on migration, IGF-I is known to stimulate proliferation of many cell types, and this is also true of cytotrophoblast proliferation in vitro (Hills et al., 2004; Li & Zhuang, 1997). The data reporting stimulation of both proliferation and migration of trophoblasts by IGF-I are contradictory as migratory trophoblasts in vivo lose their ability to proliferate as they move away from the villus and further work is needed to clarify these effects. IGF-II is expressed by EVT columns in a gradient, with the most abundant levels in trophoblasts at the leading edge of the columns in vivo (Han et al., 1996). Although IGF-II stimulates EVT migration and invasion in vitro, the mechanism by which it does this is currently unclear as it has no effect on α5 or β1 integrin subunit expression, or MMP or plasminogen activator activity (Hamilton et al., 1998; Irving & Lala, 1995a). Despite this, the upregulation of IGF-II is believed to be of greater physiological importance with respect to trophoblast outgrowth than IGF-I, as EVTs predominantly express the IGF-RII (Hamilton et al., 1998). IGF-I and IGF-II are regulated by Insulin-like growth factor binding proteins (IGFBP) which bind and modulates their mitogenic and metabolic effects (Baxter, 1997). IGFBP-1 to -6 are expressed by human decidua (Han et al., 1996), but of these IGFBP-1 has been studied in the greatest depth in this setting. IGFBP-1 is produced primarily by decidualised stromal cells, although it is also expressed by cytotrophoblasts and syncytiotrophoblast (Bell et al., 1985; Guidice, 1997; Han et al., 1996). IGFBP-1 increases trophoblast migration, possibly by blocking the interactions between α5β1 integrin and fibronectin, and increases trophoblast invasion by a non-MMP related mechanism, possibly by stimulating an alternative gelatinolytic enzyme (Bischof et al., 1998; Irving & Lala, 1995a).

The vascular endothelial growth factor (VEGF) family of ligands and receptors are key regulators of vasculogenesis and angiogenesis in the endothelium. VEGF-A and VEGF-C, along with their respective receptors VEGFR-1 and VEGFR-3 are expressed by invasive EVTs, and are believed to promote an invasive phenotype as blocking VEGF binding in vitro results in decreased expression of α1 integrin and an increase in EVT
apoptosis (Zhou et al., 2002). The expression of VEGF-A and VEGFR-1 by invasive EVTs are reduced in cases of pre-eclampsia, and therefore if a similar reduction of α1 integrin expression and increase in apoptosis occurred in vivo, this may contribute to the decreased trophoblast invasion associated with pre-eclampsia (Zhou et al., 2002). A further member of the VEGF family, placenta growth factor (PIGF), also binds to VEGFR-1 and has been shown to promote the proliferation of the EVT cell line HTR-8 (Athanassiades & Lala, 1998). However, as normal invasive EVTs do not proliferate in vivo, using a proliferative cell line to study the proliferation of EVTs is flawed, and this single study does not provide conclusive evidence that PIGF promotes trophoblast proliferation.

1.3.5.i.a TGFβ superfamily

The TGFβ group of cytokines have a variety of roles in the regulation of trophoblast differentiation. TGFβ1 is primarily expressed by the syncytiotrophoblast, with low levels of expression in the EVT cell columns and decidua (Lysiak et al., 1995). In vitro, TGFβ1 inhibits cytotrophoblast proliferation, and promotes cytotrophoblast fusion into the syncytiotrophoblast (Graham & Lala, 1991; Li & Zhuang, 1997). TGFβ3 was reported to inhibit trophoblast invasion and outgrowth as expression was observed to peak between 6 and 9 weeks of gestation, coinciding with the time of significant trophoblast invasion (Caniggia et al., 1999). Furthermore, blocking the expression or activity of TGFβ3 in human first-trimester villous explants increased the formation of EVT columns, indicative of an invasive phenotype (Caniggia et al., 1999). However, the temporal changes in TGFβ3 expression could not be repeated by Simpson and colleagues, who only detected low levels of TGFβ3 in placentae between 7 and 19 weeks of gestation, bringing the above results into question (Simpson et al., 2002).

TGFβ binds to specific cell surface receptors including the TGFβ type I and type II receptors (TGFRI and TGFRII) and the proteins that associate with these receptors, betaglycan and endoglin (Miyazono, 1997; Wrana et al., 1994). Endoglin associates with TGFR-I and TGFR-II to form a receptor complex that binds TGFβ1 and β3 isoforms with high affinity, thereby acting as a regulatory component which may modify specific responses to TGFβ (Lastres et al., 1996; Yamashita et al., 1994). Endoglin is present at
high levels on the syncytiotrophoblast, and is specifically expressed at sites of EVT column initiation (Caniggia et al., 1997). Blocking the effects of endoglin with antibodies or antisense oligonucleotides in first trimester human villous explants results in villous cytotrophoblast proliferation and significant increases in EVT outgrowth and migration, suggesting that TGFR-endoglin complexes inhibit trophoblast differentiation into an invasive phenotype (Caniggia et al., 1997).

Activin, inhibin and follistatin are all members of the TGFβ superfamily produced by the human endometrium, and have been implicated in regulating angiogenesis, decidualisation, implantation and immunomodulation at the materno-fetal interface (Florio et al., 2004). Activins and inhibins are dimeric glycoproteins, formed from different combinations of the same α and β subunits. Activins are homodimers composed of two β subunits, while inhibins are heterodimers composed of an α and a β subunit. Activin is regulated by inhibin and follistatin. Follistatin expression is significantly increased in early pregnancy and binds activin with high affinity, thereby preventing the interaction of activin with the TGFR-II (Jones et al., 2000; Shimonaka et al., 1991). Inhibin antagonizes activin by competing for binding to the TGFR-II, however as inhibin has a much lower affinity to TGFR-II than activin it may utilize proteins such as inhibin-binding protein and betaglycan to selectively enhance this interaction (Chapman & Woodruff, 2001; Lewis et al., 2000).

During decidualisation and in early pregnancy α and β activin/inhibin subunit expression is upregulated in the stromal cells of the decidua (Jones et al., 2000). In the placenta, activin A (a βA homodimer) and its receptors are expressed in first trimester syncytiotrophoblast and cytotrophoblast, and are retained during trophoblast differentiation into the EVT lineage (Caniggia et al., 1997a; Peng et al., 1999; Petraglia, 1997; Rabinovici et al., 1992; Schneider-Kolsky et al., 2002). Activin A promotes trophoblast differentiation to an invasive phenotype, and stimulates the production of paracrine agents involved in invasion (Caniggia et al., 1997a). In vitro, the addition of activin A, but not inhibin A, to first trimester chorionic villous explants stimulates trophoblast outgrowth characterized by the expression of MMP-9, MMP-2 and HLA-G, indicating an invasive phenotype (Caniggia et al., 1997). Activin A stimulation of trophoblast outgrowth can be blocked by the addition of follistatin or TGFβ3 (Caniggia...
et. al., 1997a). Therefore TGFβ3 and activin have opposing effects on trophoblast outgrowth.

Figure 1.7 – Diagram demonstrating the roles of TGFβ superfamily members involved in the regulation of trophoblast differentiation.

1.4 Physiological hypoxia in the first trimester of pregnancy

As the EVTs invade the spiral arteries early in pregnancy they form plugs which occlude the spiral arteries and prevent maternal blood from entering the IVS, thereby creating a physiologically hypoxic environment (Burton et. al., 1999; Hustin & Schaaps, 1987; Jaffe et. al., 1997). This may also benefit the developing placenta by providing physical protection from minimal pressure rises created by freely spurting maternal arterial blood which could easily displace implanting villi (Hustin & Schaaps, 1987). Trophoblasts are extremely sensitive to oxygen concentration, and evidence is accumulating to demonstrate that a low oxygen environment early in pregnancy regulates trophoblast differentiation, and may be a key aspect in the success of pregnancy (Caniggia & Winter, 2002; Hempstock et. al., 2003; Jauniaux et. al., 2000). However, there is still a level of controversy surrounding the presence of maternal blood in the IVS in the first trimester, and the effects that oxygen concentration have on trophoblast differentiation.
1.4.1 Evidence for placental hypoxia in the first trimester of pregnancy

Initially it was believed that immediately after blastocyst implantation a number of endometrial vessels are opened by trophoblast phagocytic activity and maternal blood enters the future IVS. However, in 1960 Hamilton and Boyd reported plugs of trophoblast in the spiral arteries that loosened at the end of the first trimester (Hamilton & Boyd, 1960). Hamilton and Boyd proposed that the presence of these trophoblast plugs may reduce the pressure of blood reaching the IVS (Hamilton & Boyd, 1960). That maternal blood entered the first trimester IVS was also challenged by Hustin & Schaaps (1987), who used a multifaceted approach to demonstrate that during the first trimester the growing embryo and its placenta are completely separated from the maternal uterine circulation by trophoblastic plugs, which are dislocated between 10 and 12 weeks of gestation (Hustin & Schaaps, 1987).

Hustin and Schaaps noticed during chorionic villous sampling for antenatal diagnostics that no samples were ever blood tinged or followed by blood leakage, even though the IVS was penetrated. Furthermore, hysteroscopy results showed no blood discouloration in the IVS (Hustin & Schaaps, 1987). These observations led Hustin and Schaaps to examine blood flow in the IVS by transvaginal ultrasound, where they were unable to delineate moving echoes in the IVS before week 13 of gestation in normal pregnancies. Further confirmation was obtained by perfusing the uterine arteries of hysterectomy specimens with contrast media at high pressure to determine that no contrast media could be detected in the IVS (Hustin & Schaaps, 1987).

1.4.1.i Ultrasound evidence for a lack of maternofetal circulation in the first trimester

A number of studies have provided ultrasonographic evidence to demonstrate the progressive loosening of the trophoblast plugs, exposing the developing placenta to maternal blood flow from approximately 10 weeks of gestation (Jaffe & Woods, 1993; Jauniaux et. al., 2003b; Jauniaux et. al., 1992; Schaaps et. al., 2005). After the trophoblast plugs are loosened and maternal blood is able to enter the IVS, fountain-like spurts of blood are seen from the opening of the spiral arteries onto the decidual plate with a progressive decrease of the resistance in the uterine spiral arteries from 10 to 17 weeks of gestation (Jauniaux et. al., 1992). It is likely that the onset of the maternal circulation is a
progressive phenomenon that starts at the periphery and gradually extends towards the centre of the IVS, and thus it should not be assumed that regional oxygen tension across the materno-fetal interface is uniform (Jauniaux et. al., 2003b). Furthermore, these authors have suggested that the premature appearance of maternal blood in the IVS is associated with an increased occurrence of miscarriage (Hempstock et. al., 2003; Jauniaux et. al., 2003b).

However, it is important to note that while there is a mounting body of evidence to support a low oxygen environment during the first trimester, not all ultrasound evidence supports this. Kurjak and colleagues argue that maternal blood flow into the IVS occurs early in the first trimester from 6 weeks of gestation (Kurjak & Kupesic, 1997; Kurjak et. al., 1993). In particular, these researchers conducted a large study where 270 normal pregnancies were examined by three-dimensional power Doppler ultrasonography and concluded that the number of areas in which blood penetrates the IVS increased gradually with gestation (Kurjak et. al., 1999).

While ultrasound techniques do have limitations in terms of accuracy, particularly at the low velocities of flow potentially present in the IVS, these results are just one aspect of a growing body of evidence from many areas including the direct measurements of oxygen tension, histology and in vitro studies, examining maternal blood flow and subsequent low oxygen conditions in the IVS in the first trimester.

1.4.1.ii Direct evidence for low oxygen conditions in the first trimester of pregnancy

With no maternal blood present in the IVS during the first trimester of pregnancy, implantation, organogenesis and the building of the definitive placenta all occur in low oxygen conditions, which are physiologically normal in this setting. From 8-10 weeks of gestation direct measurements of oxygen tension showed that the partial pressure of oxygen in the placenta (17.9mmHg) was significantly lower than the endometrium (39.6mmHg) (Rodesch et. al., 1992). Furthermore, from 12-13 weeks of gestation the placental oxygen tension increased to levels which were not significantly different from the endometrium, consistent with the loosening of the trophoblast plugs at that time (Rodesch et. al., 1992). More recently, Jauniaux and colleagues measured respiratory
gases and acid-base values in 30 early pregnancies and confirmed that before 11 weeks of gestation the partial pressure of oxygen in the placenta was 2.5 times lower than that in the decidua (Jauniaux et. al., 2001).

1.4.1.iii Histological evidence for low oxygen conditions in the first trimester

The presence of trophoblast plugs in the maternal spiral arteries during the first trimester of pregnancy has been confirmed by histological evidence. A histologic review of 12 early pregnancy hysterectomy specimens in the Boyd Collection confirmed that before the 8th week of pregnancy maternal arterial connections with the IVS are restricted to a torturous network of intercellular spaces with no direct channels, leading the authors to conclude that the maternal circulation to the IVS is likely to be extremely limited before this time with only plasma filtrate likely to be able to enter the IVS (Burton et. al., 1999). However, these findings have been disputed by other authors. In a larger study of 25 pregnant uteri, Meekins and colleagues report that the majority of spiral arteries are not plugged and maternal blood is present in the IVS from 8 weeks of gestation (Meekins et. al., 1997). Furthermore, Enders (1989) who examined extremely early (approximately 1 week after implantation) human implantation sites from the Carnegie collection reported that some lacunae in the trophoblastic plate were confluent with maternal vessels at the periphery of the implantation site, suggesting that maternal blood is in contact with the trophoblastic plate at the time of implantation (Enders, 1989).

1.4.1.iv In vitro evidence for low oxygen conditions in the first trimester

It is becoming evident that low oxygen conditions are required for placental development to be successful. Hyperoxic tissue damage is mediated through increased intracellular production of superoxide anions (Watson et. al., 1998). Early placental tissue is unable to protect itself against oxidative damage, as the antioxidant enzymes copper/zinc superoxide dismutase and mitochondrial superoxide dismutase are not evident in the syncytiotrophoblast until approximately 8 to 9 weeks of gestation, increasing significantly after this point at the time trophoblast plugs are thought to loosen and the placenta becomes exposed to gradually increasing oxygen tension (Watson et. al., 1997; Watson et. al., 1998). The absence of protection by superoxide dismutase enzymes during the first 8
weeks of gestation renders the syncytiotrophoblast acutely sensitive to oxygen-mediated damage.

The increase in oxygen tension at the end of the first trimester is associated with increased syncytiotrophoblast expression of the markers of oxidative stress including the inducible form of heat shock protein 70, nitrotyrosine residues and hydroxynoneal, which are markers of cellular stress, protein oxidative damage and lipid oxidative damage respectively (Jauniaux et al., 2000). Heat shock protein 70 and nitrotyrosine, but not hydroxynoneal, showed increased expression in the peripheral regions of normal placentae (Jauniaux et al., 2003b). Peripheral regions of the placentae also showed morphological signs of oxidative stress including increased electron density of the mitochondria, a thin syncytiotrophoblast layer devoid of microvilli and infrequent and flattened cytotrophoblasts (Jauniaux et al., 2003b). However, after approximately 11 weeks of gestation heat shock protein 70 and nitrotyrosine expression were of similar intensity in both peripheral and central regions of the placentae, indicating that the maternal circulation is almost fully established by this time (Jauniaux et al., 2003b).

1.4.2 Placental and fetal nutrition in the absence of maternal blood

Although the placenta appears to thrive in the absence of oxygen in the first trimester, the developing placenta and fetus would be unable to do this without sufficient nutrition. In the second and third trimesters maternal blood flow provides hemotrophic nutrition to the placenta and fetus. In order to make it feasible for the maternal blood supply to be blocked by trophoblast plugs in the first trimester an alternative source of placental nutrition must be available at this time. In the first trimester it has been shown that the placenta and fetus are supplied by histiotrophic nutrition, which arises from extracellular material derived from the endometrium and uterine glands that accumulates in the IVS between the maternal and fetal tissues (Burton et al., 2002). The maternal uterine glands remain active until at least week 10 of gestation and their secretions are delivered freely into the IVS where they are able to be used as a form of nutrition through syncytiotrophoblast phagocytosis (Burton et al., 2002). The secondary yolk sac and extraembryonic coelom (EEC) also play important roles in fetal nutrition during the first 10 weeks of gestation by synthesizing proteins and providing a preferential pathway across the villous trophoblast respectively (Burton et al., 2002; Jauniaux & Gulbis, 2000;
Jauniaux et al., 2003a). It is also possible that the diffusion of oxygen across the EEC supplies adequate oxygen to the fetus in the absence of a functional maternal or placental circulation while maintaining the embryo in a low oxygen environment (Burton et al., 2003; Jauniaux et al., 2003b).

In conclusion, although some contrary evidence exists, the lack of maternal blood flow and subsequent physiological hypoxia in the first trimester of human pregnancy is strongly supported by a growing body of physical, ultrasound, direct, histological and in vitro evidence. The developing placenta and embryo can meet all their nutritional and metabolic needs without maternal blood flow in the IVS, and in cases where such a blood flow does exist it appears to be detrimental to placental development.

1.4.3 Mechanisms of oxygen sensing by trophoblasts

In order to be able to respond to increasing levels of oxygen as the maternoplacental circulation is established at the end of the first trimester, and as invasive trophoblast progress along the spiral arteries, trophoblasts must be able to accurately sense oxygen tension (for review refer to Caniggia & Winter, 2002). The exact mechanism by which trophoblasts sense oxygen tension is currently unclear, however several potential pathways have been identified. Many of these pathways utilize the formation of reactive oxygen species (ROS), but it is currently unclear whether hypoxia results in an increase or decrease in cellular levels of ROS (DeMarco & Caniggia, 2002). In hypoxic conditions trophoblast oxygen sensing mechanisms utilize several different pathways to control gene expression. These pathways often utilize redox-sensitive transcription factors, of which the Hypoxia Inducible Factor (HIF) family are the best characterized in trophoblasts.

1.4.4 The hypoxia inducible factor family of transcription factors

To date, three members of the HIF family have been identified, but it is possible that others exist. All the members of the HIF family consist of an inducible alpha subunit (HIF-α) and a constitutively expressed beta subunit (HIF-β, also known as ARNT). Under physiologically normoxic conditions HIF-α is rapidly broken down following binding of von Hippel-Lindau tumour suppressor protein (pVHL), which targets HIF-α for degradation by the ubiquitin-proteasome pathway (Figure 1.8) (Cockman et al., 2000; Maxwell et al., 1999; Ohh et al., 2000). pVHL binds to HIF-α following hydroxylation
of proline residues in the oxygen dependent degradation domain of HIF-\(\alpha\) (Jaakkob \textit{et. al.}, 2001; Masson \textit{et. al.}, 2001). However, under hypoxic conditions this hydroxylation of the proline residues is blocked, resulting in stabilization and accumulation of HIF-\(\alpha\) in the cytoplasm (Ivan \textit{et. al.}, 2001; Lando \textit{et. al.}, 2002). The stabilized HIF-\(\alpha\) can translocate to the nucleus where it dimerizes with HIF-\(\beta\). The HIF \(\alpha\)-\(\beta\) dimer is then able to bind to DNA and induce gene expression (Wang \textit{et. al.}, 1995). Hypoxia further enhances the activity of HIF-\(\alpha\) by promoting the ability of the HIF-\(\alpha\) C-terminal trans-activation domain (CAD) to interact with co-activators such as p300 and thereby amplify HIF-\(\alpha\) induced gene transcription (Ema \textit{et. al.}, 1999).
Figure 1.8 - Regulation of HIF-1. Under hypoxic conditions HIF-1α is phosphorylated allowing it to bind HIF-β and cofactors such as p300 to induce gene transcription. The phosphorylation of HIF-1α is not solely dependent on hypoxia and the binding of growth factors to extracellular receptors can affect this process. Under normoxic conditions HIF-1α is targeted for degradation along the ubiquitin-proteasome pathway by the binding of pVHL, preventing the formation of active HIF-1. Hypoxia inhibits pVHL and FIH-1 allowing protein expression and transcriptional activity of HIF-1α. HIF is able to be regulated independently of oxygen by the binding of growth factors to receptor tyrosine kinases which can firstly induce translation of HIF-1α in order to overcome the oxygen sensor mediated HIF-1α degradation, and secondly enhance the transcriptional activity of active HIF by phosphorylation of the p300 co-activator.

1.4.4.i Hypoxia inducible factor-1

The predominant member of the HIF family is HIF-1, which has been shown to regulate the expression of more than 60 genes in a variety of cell types (Semenza, 2003). In the placenta in vivo, HIF-1α has been shown to be strongly expressed from 5 weeks of gestation, predominantly in the EVT, syncytiotrophoblast and villous cytotrophoblast (Caniggia et al., 2000; Rajakumar & Conrad, 2000). However, expression decreases at
around 9 weeks of gestation, and by 12 weeks of gestation immunoreactivity for HIF-1α is weak or absent (Caniggia \textit{et. al.}, 2000; Caniggia & Winter, 2002).

1.4.4.ii Hypoxia inducible factors 2 and 3

HIF-2 and 3 arise from combinations of HIF-2α and 3α subunits respectively with the common HIF-β chain. HIF-2α is expressed in the syncytiotrophoblast, cytotrophoblasts and mesenchymal cells of the first trimester placenta (Rajakumar & Conrad, 2000). HIF-2α mRNA increases significantly with advancing gestational age, but conversely HIF-2α protein decreases with gestational age, indicating that as for the degradation of HIF-1α by pVHL, the main point of regulation for HIF-2α is post-transcriptional (Rajakumar & Conrad, 2000). In \textit{situ}, pVHL is expressed in villous cytotrophoblast cells and sites of EVT outgrowth initiation, corresponding to cytoplasmic HIF-2α staining (Genbacev \textit{et. al.}, 2001). Confusingly, it has been reported that as trophoblasts invade into the decidua, pVHL is downregulated and HIF-2α is localized to the nucleus (Genbacev \textit{et. al.}, 2001). The upregulation of trophoblast pVHL in response to hypoxia has been confirmed \textit{in vitro} (Genbacev \textit{et. al.}, 2001). Anchoring villi with attached trophoblast columns show strong staining for pVHL when cultured in 2% oxygen, whereas in 10% or 20% oxygen staining for pVHL is weak or absent (Genbacev \textit{et. al.}, 2001). These results are contrary to expectation as higher oxygen conditions are proposed to result in the degradation of HIF-2α by pVHL, therefore pVHL would be expected to be downregulated in hypoxic conditions. However, it seems likely that the ratio of HIF-2α to pVHL is more important than the absolute levels of either protein in determining a cells response to low oxygen. HIF-2α would also be expected to be targeted for degradation when oxygen tension increased, not localized to the nucleus as observed in this study (Genbacev \textit{et. al.}, 2001). The function of HIF-2α in the placenta is yet to be elucidated. However, its coexpression at sites of EVT column initiation suggest that it may potentially play a role in alterations in trophoblast differentiation with changing oxygen concentrations. Human HIF-3α was first identified in 2001, however to date its placental expression pattern and functional role have not been reported (Hara \textit{et. al.}, 2001).
1.4.4.iii Regulation of HIF

The important role HIF may play in trophoblast function is further demonstrated by the number of interacting pathways regulating its activity. HIF-1α is able to be stabilized under normoxic conditions by a variety of growth factors and cytokines including EGF, insulin, heregulin, IGF I and II, TGFβ, and interleukin-1β (Feldser et al., 1999; Fukuda et al., 2002; Hellwig-Burgel et al., 1999; Laughner et al., 2001; Stiehl et al., 2002; Zelzer et al., 1998). However, stabilization of the HIF-1α subunit alone does not yield transcriptionally active HIF-1 (Salceda & Caro, 1997). The activation of HIF-1 involves multiple steps including, post-translational phosphorylation of the HIF-1α chain, nuclear translocation, HIF-β heterodimerization, DNA binding, recruitment of tissue-specific transcriptional cofactors and target gene trans-activation, which are under the control of a range of factors including oxygen concentration, growth factors, hormones, nutrients and cross communication with other signaling pathways allowing cell specific fine tuning of the hypoxic response (Lee et al., 2004; Wenger, 2002).

The protein expression and transcriptional activity of HIF-1α is able to be regulated independently of hypoxia via the phosphatidylinositol 3 kinase (PI3K) and/or mitogen activated protein kinase (MAPK) signal transduction pathways allowing cell or stimulus specific regulation of HIF-1α that is independent of the oxygen-mediated regulation of HIF-α levels (Conrad et al., 1999; Fukuda et al., 2002; Jiang et al., 2001; Richard et al., 1999). The MAPK pathway in particular is known to play an important role in the signal transduction of a range of cellular responses including proliferation, differentiation and stress responses (Kita et al., 2003). In human trophoblasts the PI3K signaling pathway is activated by interleukin-12, whereas the MAPK signaling pathway is known to mediate the effects of insulin, IGF-II, insulin-like growth factor binding protein -1 (IGFBP-1), leptin, endothelin-1, gonadotrophin-releasing hormone (GnRH) and epidermal growth factor (EGF) (Bifulco et al., 2003; Chakraborty et al., 2003; Gleeson et al., 2001; Kang et al., 2000; Kong et al., 2002; Mackova et al., 2003; McKinnon et al., 2001). The MAPK pathway can also be activated by the formation of ROS in hypoxic conditions (Kulisz et al., 2002).

Finally the transcriptional activity of HIF-1 can be regulated by Factor Inhibiting HIF-1 (FIH-1), an Fe(II)-dependent enzyme that uses molecular oxygen to modify its substrate,
potentially allowing FIH-1 to function directly as a cellular oxygen sensor (Mahon et al., 2001). FIH-1 acts as co-repressor by forming a complex with both pVHL and HIF-1 (Lando et al., 2002). However, to date no data on the placental expression of FIH-1 has been reported.

1.4.5 Other transcription factors involved in regulating the response of trophoblasts to hypoxia

Several other transcription factors involved in trophoblast differentiation are responsive to hypoxia (Figure 1.9). The transcription factors Id1, Mash-2 and the helix-loop-helix transcription factors Upstream Stimulatory Factor-1 and 2 (USF1 and USF2) which mediate the effects of Mash-2 are all upregulated in 2% oxygen in comparison to 20% oxygen (Jiang et al., 2000; Jiang & Mendelson, 2003). The upregulation of Mash-2, USF1 and USF2 may inhibit cytotrophoblast fusion into syncytiotrophoblast (Jiang et al., 2000; Jiang & Mendelson, 2003).

The elevation of intracellular Ca\(^{2+}\) is believed to activate an HIF-1 independent signaling pathway that involves the transcription factor activator protein-1 (AP-1), with cooperation between the HIF-1 and AP-1 pathways allowing fine regulation of hypoxic gene expression (Laderoute et al., 2002; Salnikow et al., 2002). AP-1 is a dimeric transcription factor composed from the products of the Jun and Fos proto-oncogenes (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) (Dakour et al., 1999). AP-1 transcription factors are believed to play an important role in trophoblast differentiation. In villi, AP-1 transcription factor expression is limited, however EVTs express c-Jun, JunB, c-Fos, FosB and Fra2 both in the first trimester and later in gestation (Bamberger et al., 2004). To date, no data on trophoblast intracellular calcium elevation or the role of the AP-1 pathway in mediating the effects of hypoxia in trophoblasts has been published. However, it is feasible that, as hypoxia is able to influence the AP-1 pathway in other cell types, this pathway may play a role in the response of trophoblasts to hypoxia (Schorpp-Kistner et al., 1999).
Figure 1.9 – Regulation of hypoxia-inducible (non-HIF) transcription factors. Hypoxia results in the upregulation of a number of transcription factors including the Jun subunit of AP-1 transcription factors, as well as Id1, Mash2 and USF1/2. The Jun proto-oncogenes are able to bind to other Jun subunits, or to Fos subunits to form AP-1 transcription factors which are then able to bind to one of three AP-1 transcription factor binding sites in the promoter region of hypoxia inducible target genes and induce transcription. Jun can also be upregulated independently of hypoxia by increased cellular calcium levels. Id1 and Mash2 can bind directly to the promoter region of target genes and induce transcription. The upregulation of Mash2 also results in the upregulation of USF1 and USF2 which further induce transcription of hypoxia-regulated genes by binding to E boxes in the promoter region of the target gene.

The number of pathways and potential factors involved in the response of trophoblasts to oxygen demonstrate that oxygen sensing pathways are intricately linked by a vast range of factors to trophoblast function. Therefore, changes in oxygen concentration in the first trimester should not be considered to affect trophoblast differentiation and function in an isolated manner, but rather to act as part of a regulatory network.

1.5 The trophoblast response to low oxygen in the first trimester of pregnancy

Transcription factors facilitate the expression of a wide range of genes in response to hypoxia in the first trimester of pregnancy. Gene responses to hypoxia can be broadly
divided into two groups – those that promote cell survival by increasing oxygen delivery, decreasing oxygen consumption, and adapting cellular metabolic responses and are therefore a common response to hypoxia in many tissues, and those that play a specific role in the regulation of implantation and placentation. Changes in gene expression in hypoxia that promote trophoblast survival include increased glucose consumption and the expression of proteins involved in glycolysis (Baumann et al., 2002; Hoang et al., 2001). The following sections will focus specifically on the second group of proteins and how hypoxia regulates trophoblast differentiation in the first trimester.

1.5.1 The effect of low oxygen on trophoblast differentiation

In vivo, only the EVTs most proximal to the villi proliferate (Irving & Lala, 1995a; Vivovac et al., 1995). As EVTs migrate away from the villi and invade into the maternal decidua they progressively develop an invasive phenotype and are no longer able to proliferate (Genbacev et al., 2000; Genbacev et al., 1997). There are two opposing schools of thought on the effect of hypoxia on trophoblast differentiation in the first trimester of human pregnancy. Evidence exists to demonstrate that hypoxia promotes a proliferative trophoblast phenotype, which would create a large pool of trophoblasts, thereby providing sufficient numbers to invade into the maternal decidua. However, contradictory results have also shown hypoxia to promote an invasive trophoblast phenotype which may be important in achieving sufficient depth and extent of trophoblast invasion.

1.5.2 Evidence for low oxygen promoting a proliferative phenotype in villous trophoblast

Several different lines of evidence support the hypothesis that hypoxia promotes trophoblast proliferation. In vivo, there is a high level of mitoses in villous cytotrophoblasts between 6 and 10 weeks of gestation, but the mitotic index decreases significantly between 10 and 12 weeks of gestation when the placenta is exposed to maternal blood (Tedde & Tedde-Piras, 1978). This observation is supported by in vitro evidence. Firstly, HTR-8/SVneo cells, a human first trimester cytotrophoblast cell line, show both increased proliferation and reduced invasion through Matrigel when cultured in 2% oxygen conditions (Kilburn et al., 2000). Secondly, isolated first trimester cytotrophoblasts show increased rates of DNA synthesis in 2% oxygen in comparison to
20% oxygen (Jiang et al., 2000). Thirdly, in vitro the ratio of cytotrophoblast:syncytiotrophoblast nuclei abruptly declines in placentae over 8 weeks of gestation, despite the number of cytotrophoblast nuclei per unit area remaining constant, suggesting that cytotrophoblast proliferation is greater in the hypoxic conditions of early pregnancy (Bose et al., 2003). Finally, in comparison to explants cultured in 20% oxygen, first trimester villous explants cultured in 2 or 3% oxygen show increased BrdU (thymidine analogue) incorporation, increased EVT outgrowth, and an increase in the total number of cells in this outgrowth (Caniggia et al., 2000; Genbacev et al., 1997). In support of these results, first trimester villous explants cultured in 1 % and 5% oxygen formed 55% and 40% more outgrowths respectively than explants cultured in 20% oxygen (Sferruzzi-Perri & Roberts, 2003).

Hypoxia has also been shown to reduce the invasive capacity of trophoblasts and the expression of molecules associated with an invasive trophoblast phenotype such as α1 integrin and MMP-2 (Caniggia et al., 2000a; Crocker et al., 2003; Genbacev et al., 1997; Kilburn et al., 2000). Culture in 3% oxygen also reduces the invasion of isolated cytotrophoblasts from the vessel lumen into the walls of dissected spiral arteries in comparison to culture in 20% oxygen (Crocker et al., 2003).

Further indirect evidence for the stimulation of a proliferative trophoblast phenotype in response to hypoxia is provided by the regulation of key cytokines that promote this phenotype. TGFβ3 may act under the control of HIF-1α to inhibit trophoblast invasion (Caniggia et al., 2000; Nishi et al., 2004; Schaffer et al., 2003). TGFβ3 expression in placental villi has been reported to increase from approximately 6 to 9 weeks of gestation, with a failure in its downregulation around 9 weeks of gestation reported to be associated with shallow trophoblast invasion, which may predispose the pregnancy to pre-eclampsia (Caniggia et al., 1999; Caniggia et al., 2000a). Therefore, induction of HIF-1α by hypoxia in the first trimester may upregulate TGFβ3 expression, inhibiting trophoblast differentiation to an invasive phenotype (Caniggia et al., 2000a). However, these temporal changes in TGFβ3 expression were not detected by Simpson and colleagues, who could only detect low levels of TGFβ3 immunostaining between 7 and 19 weeks of gestation (Simpson et al., 2002). In addition, IGF-II appears to be able to mediate the effects of hypoxia on extravillous outgrowth (Sferruzzi-Perri & Roberts, 2003). Addition of exogenous IGF-II to first trimester villous explant cultures increases
the formation of EVT outgrowth in 20% oxygen conditions, but not in 1% or 5% oxygen conditions in which outgrowth production is elevated independent of exogenous IGF-II (Sferruzzi-Perri & Roberts, 2003).

The identification of cytokines involved in hypoxic stimulation of trophoblast proliferation are particularly important as cytokines are able to regulate the response of trophoblasts to oxygen. Further understanding of alterations in the effect of cytokines on trophoblast differentiation in different oxygen concentrations is important to understand the multiple interactions of regulatory cytokines in the physiologically normal low oxygen conditions of pregnancy. This will lead to increased understanding on how hypoxia affects trophoblast phenotype during this time.

1.5.3 Evidence for low oxygen promoting an invasive trophoblast phenotype

In contrast to the above discussion, a smaller body of indirect evidence exists that suggests that hypoxia may induce EVT differentiation into an invasive trophoblast phenotype. One mechanism of trophoblast invasion involves urokinase-type plasminogen activator (uPA), which is secreted as an inactive pro-enzyme that is activated upon binding to its highly specific receptor (Blasi, 1993). Receptor bound uPA is able to convert plasminogen into plasmin, which is then able to degrade several ECM components, activating growth factors and latent metalloproteases required for invasion (Mayer, 1990; Petersen et. al., 1988; Saksela, 1985). In direct contrast with the results of Kilburn and colleagues (2000) discussed previously, Graham and colleagues found that culture of HTR-8/SVneo trophoblast cells in 1% oxygen increased the invasion of these cells through Matrigel in comparison to culture in 20% oxygen, by upregulating uPA receptors (Graham et. al., 1998; Graham et. al., 1999). The uPA system is regulated by PAI-1 and PAI-2, which inhibit both free and bound uPA by forming irreversible covalent complexes. HTR8/SVneo cells cultured in hypoxia show elevated PAI-1 expression (Fitzpatrick & Graham, 1998). Therefore, Graham and colleagues propose a model whereby hypoxia stimulates trophoblast invasion by increasing uPA receptor expression at the leading edge of the invading cell, as well as stimulating the secretion of PAI-1 at the receding edge where proteolytic activity is no longer required but detachment of the cell is needed for migration (Graham et. al., 2000).
In contrast to the pro-proliferative actions of TGFβ3 and IGF II cytokines and hormones have also been associated with stimulating trophoblast invasion in low oxygen conditions. Leptin is a hormone primarily produced in humans by adipocytes and has a role in the regulation of food intake and energy expenditure. However significant amounts of leptin are also produced by the placenta during pregnancy, with levels peaking in the second trimester (Masuzaki et. al., 1997). In vitro, hypoxia activates the human leptin gene promoter via HIF-1 in BeWo choriocarcinoma cells (Grosfeld et. al., 2002). Leptin is expressed homogenously throughout extravillous cell columns, the leptin receptor is expressed in a clear gradient along the EVT columns with the strongest expression at the distal cells of the columns (Castellucci et. al., 2000). Accordingly the binding of leptin to its receptor is believed to stimulate an invasive trophoblast phenotype (Castellucci et. al., 2000). However, these links are not necessarily causative and the role of hypoxia in the stimulation of leptin production and activity in the first trimester of human pregnancy has yet to be elucidated.

Alterations in the trophoblast adhesion molecule repertoire and production of ECM components by hypoxia have been linked with EVT differentiation into an invasive phenotype. HTR-8/SVneo cells cultured in hypoxic conditions show increased trophoblast fibronectin production, but decreased expression of α5 integrin which forms part of the α5β1 integrin fibronectin receptor and consequently decreased trophoblast adhesion to fibronectin (Chen & Aplin, 2003; Lash et. al., 2001). It is possible that increased trophoblast fibronectin production is an indirect result of the acidic environment that hypoxia creates (Gaus et. al., 2002). However, despite the above evidence being used by several authors as support for an invasive phenotype, fibronectin blocking antibodies do not affect trophoblast invasion in vitro, and consequently α5β1 integrin is not believed to play a significant role in trophoblast invasion (Damsky et. al., 1994). The uPA receptor may also interact directly with specific integrins, such as β1 integrin, to modulate trophoblast adhesion to, and migration towards, ECM vitronectin (Lash et. al., 2001; Wei et. al., 1996). However, PAI-1 is also able to counteract this effect by acting act as an antagonist and binding to vitronectin thereby preventing its association with the uPA receptor (Lash et. al., 2001).
1.5.4 Does the physiological hypoxia of early pregnancy promote an invasive or a proliferative trophoblast phenotype?

The confusion as to the effect of hypoxia on trophoblast differentiation in the first trimester of human pregnancy is confounded by the fact that the cellular response to hypoxia is not an isolated pathway, but a combination of multiple sensing mechanisms, transcription factors, oxidative stress responses and cytokine production, not to mention a large number of discordant results in the literature. Many of the studies providing evidence for hypoxia promoting an invasive trophoblast phenotype use the EVT hybrid cell line HTR-8/SVneo, as a substitute for EVTs which are difficult to propagate in culture, and whether this cell line truly reflects the behaviour of normal trophoblasts is open to debate. The use of explant cultures to study EVT differentiation provides significant advantages in that they allow outgrowth in a manner similar to that which occurs in vivo, but results from individual explants tend to be variable and therefore a high number of replicates are required to create meaningful data. It is interesting to note that the gene expression profile of first trimester villous explants cultured in 3% oxygen is remarkably similar to that of term placentae from high-altitude and pre-eclamptic pregnancies indicating that, although a low oxygen environment is the physiological norm in the first trimester, this model may provide insights into how a decreased oxygen supply may affect the placenta later in gestation (Soleymanlou et al., 2005). The interpretation of what EVT outgrowth from explants signifies in different explant models is also often unclear, with some reports concluding that this is indicative of EVT proliferation and clearly separate outgrowth from extravillous migration (Caniggia et al., 2000), whereas others conclude that EVT outgrowth represents trophoblast invasion (Irving et al., 1995). Finally, as the acquisition of an invasive phenotype is a progressive phenomenon we should not assume that the proliferative and invasive trophoblast phenotypes are necessarily mutually exclusive, and it is possible that the effect of hypoxia on trophoblast phenotype may vary with the specific properties of various trophoblast populations.

It is important to note that many experiments used to determine the effect of low oxygen conditions on trophoblast phenotype employ physiologically superoxic conditions of 20% oxygen \( (pO_2 = 140\text{mmHg}) \) as a control. However, once the maternofetal circulation is established the blood in the IVS is a mixture of arterial and venous blood, and blood sampled from the IVS at term has a \( pO_2 \) of 40mmHg (Howard et al., 1961).
Therefore, a more physiologically relevant control concentration of around 6-8% oxygen may more accurately represent conditions in vivo once the maternal circulation in the IVS is established. Furthermore, the range of experimental oxygen concentrations (from 1 to 3% oxygen) used to represent hypoxia may also contribute to the experimental differences observed. Finally, the gestation and mode of collection of placentae may also be important as exposure to maternal blood or significant time delay at the time of tissue collection leading to oxidative stress is able to affect immunoreactivity of not only HIF-1α, but the induction of downstream factors such as TGFβ3 (Dyson et al., 2003).

In conclusion, trophoblast differentiation is essential for the success of human pregnancy, and despite some conflicting experimental evidence, hypoxia appears to play a vital role in regulating trophoblast differentiation in the first trimester. The regulation of trophoblast differentiation by hypoxia is a result of complex interactions between factors associated with oxidative stress, oxygen sensing mechanisms and the release of inflammatory cytokines. Therefore, aberrations in any one of these factors, along with the temporal and spatial regulation of blood flow in the IVS has the potential to result in altered gene expression and trophoblast phenotype.

1.6 Summary

Cytotrophoblast differentiation down the EVT lineage in the first trimester of human pregnancy is a tightly regulated process that is essential for the success of pregnancy. This process primarily occurs at a time of pregnancy when the developing placenta exists in low oxygen conditions, and these conditions are believed to be important for placental development and trophoblast differentiation. However, the study of trophoblast differentiation during this time has been hampered by a lack of relevant models. Thus, the process of trophoblast differentiation during the first trimester is poorly understood.

1.7 Aims

The aims of this thesis were to:

1) Develop biologically relevant models by which to study cytotrophoblast differentiation
2) Use these models to further understand the process of cytotrophoblast differentiation.
3) Investigate the effect of gestation on EVT outgrowth. I hypothesise that the production of EVT outgrowth will decline with increasing gestational age.
4) Investigate the effect of oxygen on EVT outgrowth. I hypothesise that low oxygen conditions will stimulate the proliferation of EVT and their outgrowth from villous explants.
Chapter Two:

Materials and Methods
2.1 Ethical Approval

The work in this thesis investigating human tissues had the approval of the Auckland Ethics Committee, New Zealand (AKX/02/00/035).

2.2 Materials

All chemicals and organic solvents used were purchased from reputable companies and were of Analar or Laboratory Reagent grade. The composition of buffers, media and all other solutions used in this work are shown in tables 2.1, 2.2, 2.5, 2.7 and 2.8. The suppliers of fine chemicals and reagents are shown in tables 2.3 to 2.8. The antibodies, their suppliers and the dilution at which they were used in this work are shown in table 2.9. Equipment and software used in this work is shown in table 2.10.

Table 2.1 Common buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (phosphate buffered saline)</td>
<td>120mM NaCl, 2.7mM KCl, 1.5mM Na₂HPO₄, 8mM KH₂HPO₄, pH 7.4.</td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>PBS, pH 7.4 and 0.05% (v/v) Tween-20</td>
</tr>
</tbody>
</table>
### Table 2.2 Fine Chemicals and reagents used in tissue culture

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Local Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal Growth Factor</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>Insulin</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Invitrogen, Auckland</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>hCG</td>
<td>Sigma, USA</td>
<td>n/a</td>
</tr>
<tr>
<td>Streptomycin and penicillin</td>
<td>Invitrogen, Auckland</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12)</td>
<td>Invitrogen, Auckland</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>Invitrogen, Auckland</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>Matrigel</td>
<td>Becton Dickinson, Australia</td>
<td>Becton Dickinson, Auckland</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Invitrogen, Auckland</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>DNAse I</td>
<td>Roche, Germany</td>
<td>Roche, Auckland</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>Roche, Germany</td>
<td>Roche, Auckland</td>
</tr>
<tr>
<td>FGF-4</td>
<td>PeproTech, USA</td>
<td>Australian Laboratory Services, Auckland</td>
</tr>
<tr>
<td>Heparin</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
</tbody>
</table>

### Table 2.3 Tissue culture media

<table>
<thead>
<tr>
<th>Media</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Trophoblast Media</td>
<td>Dulbecco’s Modified Eagle Medium Salts/F12 (DMEM/F12) containing 10% fetal bovine serum, 5ng/mL epidermal growth factor, 5μg/mL insulin, 10μg/mL transferrin, 100μg/mL streptomycin, 20nM sodium selenite, 400U/L hCG, 100μg/mL L-glutamate and 100U/L penicillin</td>
</tr>
<tr>
<td>Conditioned Complete Trophoblast Media</td>
<td>75% Complete Trophoblast Media (as above) 25% Media pooled from cultures of villous explants incubated in a flask of Complete Trophoblast Media for 3-4 days.</td>
</tr>
<tr>
<td>Choriocarcinoma Media</td>
<td>DMEM/F12 containing 10% fetal bovine serum, 100U/L penicillin, 100μg/mL L-glutamate.</td>
</tr>
<tr>
<td>Solution/Reagent</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Tissue cryoembedding medium</td>
<td>Jung, Germany</td>
</tr>
<tr>
<td>2-methyl butane 95%</td>
<td>Acros Organics, Belgium</td>
</tr>
<tr>
<td>Microscope coverslips</td>
<td>BioLab Scientific, Auckland</td>
</tr>
<tr>
<td>Pre-cleaned microscope slides</td>
<td>BioLab Scientific, Auckland</td>
</tr>
<tr>
<td>Aquamount</td>
<td>BDH, Auckland</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>Xylene</td>
<td>BDH, Auckland</td>
</tr>
<tr>
<td>Normal goat serum</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>DAKO pen</td>
<td>DAKO, Denmark</td>
</tr>
<tr>
<td>1L Blood bags</td>
<td>Baldwin Medical, Australia</td>
</tr>
<tr>
<td>Solution/Reagent</td>
<td>Composition or Manufacturer</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Acetone</td>
<td>BDH, Auckland</td>
</tr>
<tr>
<td>Methanol</td>
<td>BDH, Auckland</td>
</tr>
<tr>
<td>Paraformaldehyde fixation solution</td>
<td>4% (w/v) paraformaldehyde in PBS, pH 7.4.</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>Blocking solution</td>
<td>10% normal goat serum in PBS</td>
</tr>
<tr>
<td>Quenching solution</td>
<td>5% H₂O₂ in methanol</td>
</tr>
<tr>
<td>Histostain-Plus Kit (broad spectrum)</td>
<td>Contains: 1) biotinylated secondary antibody reactive with rabbit and mouse IgG and IgM antibodies. 2) Streptavidin-horseradish peroxidase.</td>
</tr>
<tr>
<td>3,3-diaminobenzidene (DAB)</td>
<td>0.1% (w/v) DAB, 0.001% (v/v) H₂O₂ in PBS</td>
</tr>
<tr>
<td>Amino ethyl carbolyl (AEC+) substrate-chromogen</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>Haematoxylin (Gills No. 2)</td>
<td>Surgipath, USA</td>
</tr>
<tr>
<td>R-Phycoerythrin streptavidin</td>
<td>Jackson ImmunoResearch, USA</td>
</tr>
<tr>
<td>FGFR-2 blocking peptide</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>1:500</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1:500</td>
</tr>
<tr>
<td>HLA-G (clone MEM-G/9)</td>
<td>1:200</td>
</tr>
<tr>
<td>MHC-1 (W632)</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen (PCNA)</td>
<td>1:250</td>
</tr>
<tr>
<td>Ki67</td>
<td>1:100</td>
</tr>
<tr>
<td>M30 Cytodeath</td>
<td>1:20</td>
</tr>
<tr>
<td>Activated Caspase-3</td>
<td>1:200</td>
</tr>
<tr>
<td>α1 Integrin</td>
<td>1:66</td>
</tr>
<tr>
<td>p27</td>
<td>1:50</td>
</tr>
<tr>
<td>CD9</td>
<td>1:100</td>
</tr>
<tr>
<td>αVβ6 Integrin</td>
<td>1:25</td>
</tr>
<tr>
<td>Tenascin</td>
<td>1:100</td>
</tr>
<tr>
<td>CD45</td>
<td>1:100</td>
</tr>
<tr>
<td>Jab-1</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescin (FITC) tagged Cytokeratin</td>
<td>1:100</td>
</tr>
<tr>
<td>Bo1D11</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>G11</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FGFR-2 (clone Bek(C17))</td>
<td>1:33</td>
</tr>
</tbody>
</table>
### Table 2.7 Viability assessment reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Local Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-chloromethylfluorescin diacetate (CMFDA)</td>
<td>Molecular Probes, USA</td>
<td>Invitrogen, Auckland</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>Sigma, Auckland</td>
<td>Sigma, Auckland</td>
</tr>
<tr>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)</td>
<td>Merck, Germany</td>
<td>Merck, Auckland</td>
</tr>
</tbody>
</table>

### Table 2.8 DNA extraction and agarose gel solutions

<table>
<thead>
<tr>
<th>Solution/Reagent</th>
<th>Composition or Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>4M guanidine thiocyanate, 1% N-lauryl sarcosyl, 10mM dithiothreitol</td>
</tr>
<tr>
<td>Silica, fumed</td>
<td>Sigma, Australia</td>
</tr>
<tr>
<td>Washing buffer</td>
<td>70% ethanol, 10mM EDTA, 10mM Tris-HCl, pH8.0</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>10mM Tris-HCl, 0.1mM EDTA, pH8.8</td>
</tr>
<tr>
<td>Agarose</td>
<td>Sigma, Australia</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40mM Tris-HCl, 20mM glacial acetic acid, 1mM EDTA in H2O</td>
</tr>
<tr>
<td>Gel Loading Dye</td>
<td>0.25% xylene cyanol, 30% glycerol in H2O</td>
</tr>
<tr>
<td>Solution/Reagent</td>
<td>Composition or Manufacturer</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Laemmlli Sample Buffer</td>
<td>0.125M Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 0.04% (w/v) bromophenyl blue, pH 6.8.</td>
</tr>
<tr>
<td>Collagenase II Stock Solution</td>
<td>0.1mg/mL (Worthington Biochemical Corporation, New Jersey, USA).</td>
</tr>
<tr>
<td>Precision protein marker standard</td>
<td>Biorad, Auckland</td>
</tr>
<tr>
<td>Triton-X</td>
<td>BDH, Auckland</td>
</tr>
<tr>
<td>Zymography Developing Buffer</td>
<td>0.05M Tris-HCl, 5mM CaCl₂, 0.02% (w/v) NaN₃, pH 8.8. (prepared fresh).</td>
</tr>
<tr>
<td>Tris-Glycine Running Buffer</td>
<td>0.025M Tris, 0.192M Glycine, 0.1% SDS, pH 8.3 (do not adjust pH).</td>
</tr>
<tr>
<td>Fix Solution/De-stain solution</td>
<td>45% ethanol, 10% acetic acid in H₂O</td>
</tr>
<tr>
<td>Coomassie Blue Stain</td>
<td>0.05% Coomassie R250 (w/v), 0.05% Coomassie G250 (w/v), 50% ethanol (v/v), 10% acetic acid (v/v)</td>
</tr>
<tr>
<td>Equipment/Software</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Leica CM 1900 Cryostat</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>IEC Centra GP8R Centrifuge</td>
<td>IEC, Switzerland</td>
</tr>
<tr>
<td>Culture Incubator</td>
<td>Forma Scientific, USA</td>
</tr>
<tr>
<td>1% O₂, 5% CO₂, 94% N₂ beta standard gas mix</td>
<td>BOC, Auckland</td>
</tr>
<tr>
<td>(recipe MB83478)</td>
<td></td>
</tr>
<tr>
<td>8% O₂, 5% CO₂, 87% N₂ beta standard gas mix</td>
<td>BOC, Auckland</td>
</tr>
<tr>
<td>(recipe MB83076)</td>
<td></td>
</tr>
<tr>
<td>Fyrite Oxygen Cylinder (1-7%)</td>
<td>Bacharach, USA</td>
</tr>
<tr>
<td>TEW heat impulse sealer (TISH-300)</td>
<td></td>
</tr>
<tr>
<td>Powerpac 200</td>
<td>Biorad, Auckland</td>
</tr>
<tr>
<td>Mini-Protean II gel electrophoresis system</td>
<td>Biorad, Auckland</td>
</tr>
<tr>
<td>Nikon EL WD 0.3 Phase Contrast microscope</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Coolpix 990 digital camera</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Nikon Eclipse E400 light microscope</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Leica TC SP2 confocal microscope</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>Leica Confocal Software Version 2.5.110</td>
<td>Leica, Germany</td>
</tr>
<tr>
<td>Nikon TE2000E inverted fluorescent microscope</td>
<td>Nikon, Japan</td>
</tr>
<tr>
<td>Microsoft PhotoEditor v3.0.2.3</td>
<td>Microsoft, USA</td>
</tr>
<tr>
<td>Adobe Photoshop 5.0</td>
<td>Adobe, USA</td>
</tr>
<tr>
<td>QuantityOne Software</td>
<td>Biorad, USA</td>
</tr>
<tr>
<td>Image J</td>
<td>NIH, USA</td>
</tr>
<tr>
<td>Microsoft Excel 2002</td>
<td>Microsoft, USA</td>
</tr>
<tr>
<td>WinSTAT for Excel</td>
<td>R. Fitch Software, Germany</td>
</tr>
</tbody>
</table>
2.3 Participants and tissue collection

First trimester placentae were anonymously donated by patients undergoing elective surgical termination of pregnancy at Epsom Day Unit, Greenlane Hospital, Auckland, or Auckland Medical Aid Centre, Mt Eden, Auckland. The gestational age and fetal viability of all pregnancies prior to termination were confirmed by ultrasound assessment. Each placenta was separated from the products of conception, rinsed in sterile PBS and stored in fresh PBS at 37°C until use.

2.4 Methods

2.4.1 First trimester villous explant cultures

To study EVT outgrowth from first trimester placental villi the following explant culture system was established. Matrigel was thawed slowly at 4°C and diluted 1:10 in Dulbecco’s Modified Eagle Medium Salts/F12 at 4°C (DMEM/F12). Wells of sterile 96 well culture plates (Falcon, Australia) were coated with 50\(\mu\)L/well of 10% Matrigel and incubated at 37°C for 25 minutes. Excess Matrigel was removed, leaving a thin coat on each well. Villus tips were gently teased from placentae, separated into pieces of approximately 8 mg wet weight, and placed in the centre of each well. The plates were incubated at 37°C for 5 minutes, and then 150\(\mu\)L/well of Complete Trophoblast Medium (Table 2.3) was added. The plates were then centrifuged at 210g for 1 minute in order to facilitate contact between the explant and the Matrigel. The explants were cultured at 37°C in a humidified ambient oxygen atmosphere with 5% CO\(_2\), or in blood bags with 8% or 1.5% oxygen with 5% CO\(_2\) in N\(_2\) (as described in Appendix A). Outgrowths of trophoblasts from the explants across the thin layer of Matrigel was observed directly by phase contrast microscopy.

2.4.2 In situ immunocytochemistry

Immunocytochemistry was performed in situ on cells or explants with EVT outgrowths in the wells of 96 well plates. Media was removed from each well and cells were fixed in 100\(\mu\)L of methanol for 10 minutes. Wells were washed once with 100\(\mu\)L of PBS-tween then non-specific binding was blocked by the addition of 100\(\mu\)L of blocking solution (Table 2.5) for 10 minutes at room temperature. Wells were then washed 3 times with PBS-tween. The relevant primary antibody diluted in blocking solution was added to the
wells for 1 hour at room temperature. Control wells were incubated without addition of primary antibody. Wells were then washed 3 times with PBS-tween and endogenous-peroxidase activity was quenched by the addition of 50μL of quenching solution (table 2.5) for 5 minutes. Wells were then washed 3 times with PBS-tween. A Zymed Histostain-Plus Kit containing biotinylated secondary antibody and enzyme conjugated to streptavidin was used according to the manufacturers’ instructions and added to the wells for 10 minutes (Table 2.5). Wells were washed 3 times with PBS-tween and staining was developed with AEC+ or DAB solution for 10-20 minutes. Wells were then washed with de-ionised water. Unless the relevant primary antibody was reactive with a nuclear antigen, 100μL of haematoxylin nuclear stain was added to each well for 4 minutes. Wells were washed with tap water. Staining was observed immediately by phase contrast microscopy and digitally photographed.

2.4.3 Immunohistochemistry

2.4.3.i Preparation of thin sections of fresh frozen tissue on slides
First trimester placental tissue was divided into pieces of approximately 4mm³ at the time of collection, or explants were removed from cultures at 4, 8, 24, 48 or 96 hours. Tissue was immersed in cryoembedding medium contained in tin-foil which were floated in caps of isopentane and frozen in liquid nitrogen. A cryostat was used to cut 5μm sections from the blocks which were collected on glass microscope slides. Slides were dipped in de-ionized H₂O for 3 seconds, air dried, then fixed by immersion in cold acetone for 10 minutes. The slides were then air dried, wrapped in aluminium foil and stored at –20ºC until use. At the time of use slides were thawed and sections were circled with a DAKO pen.

2.4.3.ii Preparation of thin sections of paraffin-embedded tissue on slides
Both fresh villous tissue and outgrowth producing explants cultured in 8% oxygen were fixed in paraformaldehyde for 1hr. Explants were then washed two times in 70% ethanol, and stored in 70% ethanol until they were embedded in paraffin blocks. 6μm sections were cut on a microtome and captured on slides coated with poly-L-lysine, then incubated at 60ºC overnight to remove the wax. Prior to staining, slides were placed in xylene for 10 minutes, then in fresh xylene for a further 15 minutes to remove the wax.
The tissues were then rehydrated by incubating them in baths containing a series of sequential solutions of 100%, 95%, 70%, 50% or 30% ethanol in water (v/v) for 2 minutes in each solution. Slides were then left in de-ionised water for a further 4 minutes. Antigen retrieval was performed by circling sections with DAKO pen and covering each section with 100μL of 0.005% pepsin. Sections were then incubated at 37°C for 15 minutes, then at room temperature for a further 10 minutes. Slides were then washed in PBS-tween and ready for immunohistochemistry.

2.4.3.iii Immunohistochemistry on thin sections of tissue on slides

Non-specific binding of tissue sections on slides was blocked by the addition of 100μL of blocking solution for 10 minutes at room temperature. The slides were then washed three times with PBS-tween and covered with 100μL of the relevant primary antibody diluted in blocking solution for 1 hour at room temperature. The slides were then washed three times with PBS-tween and endogenous-peroxidase activity was removed by the addition of 50μL quenching solution for 5 minutes. The slides were then washed 3 times with PBS-tween. A Zymed Histostain-Plus Kit (Table 2.5) containing biotinylated secondary antibody and enzyme conjugate was used according to the manufacturers’ instructions. Staining was developed by incubating with AEC+ at room temperature until suitable staining had developed, usually between 10 and 30 minutes. Slides were then washed with de-ionized H₂O, immersed in haematoxylin nuclear stain for 3 seconds, then washed with tap water and coverslips mounted with Aquamount. Slides were observed by light microscopy and photographed.

2.4.4 Morphological examination of extravillous trophoblast outgrowths

Microscopic examination of EVT outgrowth from explants revealed that EVTs had a distinct morphology which could be used to recognize them by phase contrast microscopy and discern them from the infrequent fibroblast outgrowths that can occur in this model. In comparison to vimentin positive/cytokeratin negative fibroblasts, cytokeratin positive/vimentin negative EVTs growing on the Matrigel were larger, wider and had a prominent nucleus with distinctive peri-nuclear granules.
2.4.5 Detection of matrix metalloprotease activity by gelatin zymography

The activity of MMP-2 and -9 in explant culture media was examined by gelatin zymography. Media from wells containing explants that did, or wells that did not, produce EVT outgrowth was removed on day 5 of culture and diluted 1:10 in de-ionised H$_2$O. The diluted media from each sample well was further diluted in an equal volume of Laemmli sample buffer (Table 2.9) and loaded onto a 10% polyacrylamide gel containing 0.1% gelatin, and electrophoresed at 100V until the electrophoretic front was within 1cm of the bottom of the gel. A sample containing 0.5µg/mL collagenase II was included as a positive control and precision protein marker standards were run down each gel to allow molecular weight determination. The zymogram was incubated with 40mL of 2.5% Triton X-100 on a rocker at room temperature for 15mins. All but 2mL of the Triton X-100 solution was then removed and gels were incubated with 40mL of developing buffer (Table 2.9) for 15 minutes on a rocker at room temperature, then at 37°C overnight for 20 hours. The next day the developing buffer was removed and the gels were fixed (Table 2.9) for 15mins. Gels were stained with coomassie blue solution overnight, then destained briefly and left to rehydrate in de-ionized H$_2$O. MMP activity appeared as clear bands in the blue background of the zymogram. The clearance zones were quantified using a scanning densitometer and Quantity-One software (refer to Appendix A for detail on the analysis of this system).

2.4.6 Examination of cell viability in villous explants by confocal microscopy

In order to determine cell viability, explants were labeled simultaneously with the fluorescent markers 5-chloromethylfluorescein diacetate (CMFDA), which is actively taken up and metabolised into a fluorescent green compound in the cytoplasm of viable cells, and Ethidium Bromide (EtBr) which is able to enter cells in which membrane permeability has been compromised and intercollate into the DNA, thereby staining the cell nucleus fluorescent red. 164 explants from 12 placentae of 8 to 12 weeks of gestation were incubated with 5µM CMFDA in Complete Trophoblast Media at 37°C for 1½ hrs. Excess CMFDA was removed by washing the explants 3 times with Complete Trophoblast Media. Explants were then incubated with 2.5µg/mL EtBr in PBS at room temperature for 1 minute, and washed 4 times with PBS. At each timepoint one additional explant was incubated in 5% (w/v) Virkon (Biolab, Auckland) for 10 minutes
before staining to serve as a positive control of cell death. Jeg-3 choriocarcinoma cells in exponential growth were used as a control to indicate cell viability. Explants were visualized at room temperature by confocal microscopy.

2.4.7 Examination of cell viability in villous explants by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To examine cell viability at earlier timepoints and confirm the methodology used in 2.4.6, explants were stained with an alternative marker of cell viability, MTT, which is metabolised by viable mitochondria to form an insoluble purple compound. Fresh tissue from three placentae of 8 weeks of gestation was divided into explants which were incubated with 200μL/well of Complete Trophoblast Media in a 96 well plate. At hourly intervals up to 5 hours from the establishment of culture 20μL of 5mg/mL MTT was added to 3 wells for 1 hour. Liquid nitrogen was then used to freeze MTT treated explants in cryoembedding medium (as in Section 2.4.3.i). The frozen tissue blocks were cut into 5μm sections using a cryostat and collected on glass slides. Slides were fixed in paraformaldehyde for 3 minutes and air dried for 1 hour. Slides were mounted with Aquamount and visualized immediately at room temperature by light microscopy.

2.4.8 Detection of DNA laddering by agarose gel electrophoresis

In order to confirm that cells in the placental explants were undergoing apoptosis the explants were examined for DNA laddering, a marker of apoptosis. DNA was extracted from placental explants after 0, 48 and 96hrs of culture. 25 explants from each timepoint were homogenized with 500μL lysis buffer (table 2.8) and incubated in a 37°C water bath for 30 minutes to settle. DNA was extracted from the homogenate by the method of Daniel et al., 1999 (Daniel et al., 1999). Briefly, 15μL of 40mg/mL silica was mixed vigorously with the lysed cells for 1 minute. Carrier bound DNA was then pelleted by a centrifugation for 10 seconds at 10000g. The supernatant was discarded and the pellet resuspended in 1mL of freshly prepared washing buffer, then re-centrifuged and the washing step repeated. Following removal of the second wash, DNA was left to dry on the bench overnight. DNA was eluted by vortexing the pellet with 150μL of elution buffer (table 2.8) warmed to 60°C, and centrifuged at 1200g for 1 minute. The DNA containing supernatant was transferred to a fresh tube where it was stored at 4°C, or at -
20°C for prolonged storage. In order to visualize DNA laddering, 25μL of DNA extract and 3μL of dye were loaded onto a 2% (w/v) agarose gel and electrophoresed at 100V. A positive control of DNA extracted by the same method from U937 cells that had been incubated with 5μM camptothecin for 5hrs at 37°C, to induce apoptosis, was included on all gels. The gels were stained in 0.5μg/mL EtBr in TAE buffer for 20 minutes, rinsed twice in water for 10 minutes and visualized on a UV lightbox.

2.4.9 Sequential passage cultures of villous explants

Villous explants were passaged through sequential cultures in order to examine their ability to produce EVT outgrowth in extended culture without being affected by deterioration of the culture conditions. 384 explants from four placentae (two each of 9 and 11 weeks gestation) were cultured using the methods described in 2.4.1 (primary passage culture). On the seventh day of culture, villous explants were transplanted into a secondary passage culture by gently drawing individual explants into the end of a micropipette tip and placing them into wells of a fresh Matrigel-coated plate. 150μL of fresh complete trophoblast media was added and the culture was continued for seven days. Villous explants from the secondary passage culture were transplanted by the same method to a tertiary passage culture and the culture continued for seven days. The outgrowth of EVTs from the explants in each passage was observed directly by phase contrast micrography and recorded on days 1, 2, 3, 4 and 7 of each passage culture by photomicrography.

2.4.10 Isolation of trophoblasts from villous tissue after extended culture

Villus tips were gently teased from first trimester placentae from 6 to 10 weeks of gestation (as for explant culture described in 2.4.1). Flasks (t25, Falcon, Sydney) were coated with 2.5mL of 10% Matrigel and incubated at 37°C for 25 minutes. Excess Matrigel was removed from the flask, leaving a thin Matrigel coat over the bottom of the flask. Up to 200 villous explants (approximately 8mg wet weight) were randomly selected from each placenta. 15mL of Complete Trophoblast Medium was added and then explants in the media were transferred to the Matrigel coated flask and cultured for 10 days at 37°C in a humidified ambient oxygen atmosphere with 5% CO₂. The culture media was replaced once during this period on day 3 or 4 of culture. Media removed at
this time was filtered and stored at -20°C to be used in Conditioned Complete Trophoblast Media (table 2.3).

On day 10 of culture, flasks were agitated to dislodge explants that may have adhered to the Matrigel, and the explants were transferred from the flask to a 30mL universal tube (Falcon, Australia) and centrifuged for 8 minutes at 480 x g. The supernatant was removed and 20mL of warm 0.25% trypsin in PBS and 0.2mL of DNase (4000U/mL) was added. Explants were incubated with the trypsin and DNase at 37°C for 10 minutes and the tube was agitated every 2 minutes during this time. At the end of the 10 minute period explants were allowed to settle to the bottom of the tube and the supernatant was aspirated. Fresh trypsin and DNase was added and this process was repeated a further 4 times. The first digest supernatant was discarded, but the remaining 4 digest supernatants were collected and filtered through cheese cloth. 1mL of FBS and 1000U of trypsin inhibitor were then added to each supernatant stored at 37°C until all the subsequent supernatants had been collected. Supernatants were then centrifuged for 8 minutes at 480 x g. Cell pellets from the four digest supernatants were pooled and resuspended in Conditioned Compete Trophoblast Media at approximately 10,000 cells/mL. Between 50,000 and 80,000 viable cells were usually obtained from each placenta by this method. Cells were cultured on 96 well plates coated with an extremely thin layer of Matrigel (as in 2.4.1). After 24 hours residual cell debris was rinsed off with PBS and the tightly adherent cells were cultured in fresh Conditioned Complete Trophoblast Media.

2.4.11 Quantification of isolated trophoblast purity

 Cultures of cells obtained by trypsin digestion (section 2.4.10), which had undergone immunocytochemistry with antibodies reactive with cytokeratin or vimentin (as in section 2.4.2) were examined by phase contrast microscopy, and the number of cytokeratin positive and cytokeratin negative (haematoxylin stained only) cells, as well as the number of vimentin positive and vimentin negative (haematoxylin stained only) cells from each placenta were counted in 5 fields of view at 10x magnification. In order to clarify the overlap in vimentin and cytokeratin positive cells observed by immunocytochemistry, cells derived from the digestion of 3 placentae and cultured for 5 days were fluorescently double labeled with antibodies reactive with cytokeratin and vimentin and the number of
cytokeratin positive and vimentin positive cells in 10 fields of view at 10x magnification was counted.

2.4.12 Culture of villous explants with exogenous FGF-4

Explants from 6 placentae ranging from 6 to 10 weeks of gestation were placed into culture using the methodology described in section 2.4.1. Explants (at least 48 in each treatment) were incubated with either complete trophoblast media alone, or complete trophoblast media with 25ng/mL FGF-4 and 1µg/mL Heparin. All explants were then cultured in 1.5% oxygen for 5 days. On day 5 of culture, plates were removed from the gas bags and outgrowths were observed by phase contrast microscopy, and the frequency of extravillous trophoblast outgrowth recorded and analysed as described in section 2.4.14.

In order to examine the whether FGF-4 affected the viability of cells in villous explants, at least 4 explants from each treatment were removed from the culture wells after 96 hours of culture and stained with CMFDA and EtBr as in 2.4.6. In addition 3 control explants from the same placenta cultured in complete trophoblast media in 20% oxygen were stained. Explants were visualized at room temperature by confocal microscopy.

2.4.13 Quantification of the effect of low oxygen and gestational age on extravillous trophoblast outgrowth

In order to determine the effect of gestational age on EVT outgrowth production from first trimester villous explants, 3963 villous explants from 25 placentae (8 to 12 weeks of gestation) were placed in explant culture (as in section 2.4.1). Each placenta was divided into two culture plates containing at least 75 explants each. Plates were cultured at 37°C in blood bags containing either 8% or 1.5% oxygen with 5% CO₂ in N₂ (the development of this culture system is detailed in Appendix B). Bags were flushed with fresh gas mix daily in order to maintain the oxygen concentration. To quantify the effect of oxygen concentration and gestational age on the formation of EVT outgrowth both the frequency of explants that produced outgrowth and the area of the trophoblast outgrowth from explants cultured in both 1.5% and 8% oxygen conditions were measured. Data analysis was performed using Microsoft Excel 2002, and WinSTAT for Excel.


2.4.13.1 Statistical analysis of outgrowth frequency

For each culture the number of explants that had produced EVT outgrowth by day five of culture was observed by phase contrast micrography and recorded. Outgrowth frequencies were normally distributed as determined by a Kolmogorov-Smirnov test (p<0.001). A dependent Student t-test was used to calculate the statistical significance of the difference in outgrowth frequency between the two oxygen conditions. A two-sample Student t-test assuming equal variances was used to determine the statistical significance of the difference in outgrowth percentages between 1.5% and 8% oxygen conditions for data under and over 11 weeks of gestation. P-values less than 0.05 were considered to be significant.

2.4.13.ii Measurement of the area of extravillous trophoblast outgrowths

In order to be able to quantify the size of EVT outgrowth in this model, first trimester villous explants are grown on an extremely thin layer of Matrigel so that outgrowth forms in a two-dimensional layer across the Matrigel surface. EVT outgrowths were observed on day five of culture by phase contrast micrography using an inverted microscope and digitally photographed. Overlapping images were aligned using Adobe Photoshop 5.0 (Figure 2.1). It was not possible for the imaging software used to automatically detect the edge of the outgrowth due to the profile of colours contained in the phase-contrast images of EVT outgrowth, the presence of occasional red blood cells in the culture well, and the presence of the villous to which the outgrowth remained attached. Therefore, areas of trophoblast outgrowth were measured by manually tracing around the edge of the EVT outgrowth using Image J software. The accuracy of the manual tracing of outgrowth area was determined by tracing around a sample of 12 outgrowths in replicates of 4. The standard errors of the sets of replicates ranged from 0.65% to 2.94%.

The data generated from the measurement of outgrowth areas were not normally distributed as determined by a Kolmogorov-Smirnov test for normal distribution (p>0.9). Therefore, a Mann-Whitney U-test was used to calculate the statistical significance of this data. P-values less than 0.05 were considered to be significant.
2.4.13.iii Measurement of the area of individual extravillous trophoblasts in outgrowths

In order to determine if the area of individual EVTs in outgrowths was affected by oxygen concentration, the area of 800 cells from both 1.5% and 8% oxygen cultures was measured from phase contrast images obtained in section 2.4.13.ii above by manually tracing around the edge of each cell using Image J software. The 800 cells measured in each oxygen concentration were evenly divided between proximal (within the first 4 cell layers from the villus) and distal (trophoblasts from the middle to the edge of the outgrowth) regions of the cell columns. A Kolmogorov-Smirnov test confirmed that the data were normally distributed (p<0.01). A two sample t-test assuming equal variances was used to determine the statistical significance between groups.

2.4.14 Quantification of the effect of low oxygen on markers of extravillous trophoblast differentiation

Expression of α1 integrin and HLA-G was determined by immunocytochemistry and analyzed semi-quantitatively by expression in each outgrowth being rated as absent (0), weak intensity (1), medium intensity (2) or very strong intensity (3). A Mann-Whitney U-test was used to determine the statistical significance of the intensity ratings between
1.5\% and 8\% oxygen. The statistical significance of the number of outgrowths with p27 expression was determined by a Fishers Exact test.
Chapter Three:

Characterisation of an *In Vitro* Model of First Trimester Trophoblast Outgrowth
3.1 Introduction and rationale

Outgrowth of EVTs from the villous tips of first trimester placentae followed by invasion of EVTs into the decidua and transformation of the spiral arteries is essential for the success of pregnancy. It is commonly accepted that EVT outgrowth is formed by the differentiation of bipotent villous cytotrophoblasts which are capable of differentiating into either EVTs or syncytiotrophoblast (Benirschke & Kaufmann, 2000). However, the factors which direct villous cytotrophoblast differentiation into either of these lineages remain unclear. In order to study EVT differentiation in the first trimester a villous explant model was developed in which EVTs grow out from villous tips in a two-dimensional sheet across an extremely thin layer of Matrigel in vitro (James, 2001). As trophoblast differentiation is believed to be tightly regulated by an intricate network of factors specific to the villous microenvironment and the paracrine relationships it contains, it is important to maintain the structural integrity of the placental tissue while studying of this process. However, the viability of villous explants in culture is often only examined crudely, for example by measuring hCG production, thymidine incorporation or MTT metabolism, which gives measures of overall cell survival but which doesn’t give information about specific cell types in the explants. In order to use my newly developed villous explant model to study cytotrophoblast differentiation accurately it was important to determine the viability of all cell types in the explants to eliminate cell death as a confounding factor in the results. The objective of the work in this chapter was to further characterize this in vitro villous explant model in order to determine the characteristics of the EVTs in outgrowths, the viability of the explants in culture and the effect of gestational age on EVT outgrowth production.

3.2 Characterisation of cellular outgrowths from first trimester villous explants

The method of explant culture is described in detail in section 2.4.1. Briefly, wells of a 96 well plate were coated with an extremely thin layer of diluted Matrigel. Villous explants of approximately 8mg were selected at random from placentae of 8-12 weeks gestation and cultured on the Matrigel. In an atmosphere of 20% oxygen 24.7% (102/413) of explants produced sheets of cellular outgrowth where the villous tips contacted the Matrigel. These outgrowths were characterized in order to establish that they consisted solely of EVTs, and to characterise the phenotype of EVTs in the outgrowths formed in vitro.
3.2.1 Outgrowths consist entirely of trophoblasts

In order to confirm that cells in the outgrowths from the villous explants were trophoblasts, outgrowths from placental explants were stained \textit{in situ} with antibodies to cytokeratin-7. Staining of outgrowths (n = 22, from 3 placentae) with anti-cytokeratin showed that the outgrowths consisted solely of trophoblasts (Figure 3.1). That cells in the outgrowths were trophoblasts was further confirmed by their failure to stain with anti-vimentin (n = 18, from 3 placentae) (Figure 3.1).
Figure 3.1 – Photomicrographs of cellular outgrowths from first trimester villous explants stained with antibodies reactive with either cytokeratin (A) or vimentin (B). Controls were trophoblast-derived Jeg-3 choriocarcinoma cells stained with antibodies reactive with cytokeratin (C) or vimentin (D) and cryo-sectioned first trimester villous tissue stained with antibodies reactive with cytokeratin (E) or vimentin (F).
3.2.2 Cells in outgrowths from first trimester villous explants express HLA-G.

In order to further confirm that the trophoblasts present in outgrowths in this model were phenotypically similar to EVTs in vivo, the expression of HLA-G in EVTs in outgrowths was examined using antibodies specific for HLA-G, as well as W6/32 which recognizes a public determinant on all class I HLA molecules including HLA-G. HLA-G (n = 8) and the W6/32 epitope (n = 7, from 3 placentae) were expressed throughout EVT columns in all outgrowths examined (Figure 3.2).
Figure 3.2 – Photomicrographs of cellular outgrowths from first trimester villous explants stained with antibodies against HLA-G (A) or MHC class I proteins (W6/32 antibody) (B). HLA-G and Class I MHC were expressed in EVTs in first trimester villous tissue (C & D respectively). EVT outgrowths were stained with cytokeratin (E, positive control) and a class matched negative control (F).
3.3 Proliferation of trophoblasts in extravillous trophoblast outgrowths from first trimester villi

In order to examine whether EVT outgrowth was expanding due to the proliferation of cells in the outgrowth, explants and outgrowth were fixed in situ on the Matrigel coated surface of the culture plate where they were grown, and examined for the expression of two proliferation markers – Ki67 and proliferating cell nuclear antigen (PCNA) by immunohistochemistry. Ki67 is an antigen expressed in the granular components of the nucleolus during late G1, S, G2 and M phases of cell division and as it has a biological half life of less than one hour, it is only expressed in cells that are undergoing cell division (Gerdes et. al., 1984; Schrape et. al., 1987). Whereas PCNA is elevated in the nucleus during late G1 phase immediately before the onset of DNA synthesis, becoming maximal during S-phase and declining during G2 and M phases (Takasaki et. al., 1981).

Proliferation of EVTs in outgrowths of culture was examined after 3 days in culture by in situ immunohistochemistry using antibodies reactive with Ki67 and PCNA. Ki67 expression could not be detected in any of the EVTs visible on the Matrigel surface in outgrowths from villous explants (Figure 3.3). However, nuclear Ki67 staining was observed in control Jeg-3 and KGN cell lines (Figure 3.3). PCNA was expressed in the nucleus of all EVTs throughout outgrowths, and in all nuclei of control Jeg-3 cell lines (Figure 3.4).
Figure 3.3 - Photomicrographs of A) EVT outgrowth in situ stained with antibodies reactive with Ki67. Positive controls of B) Jeg3 choriocarcinomas and C) an epithelial cell line KGN in exponential growth, show nuclear expression of Ki67 in actively proliferating cells. D) Control KGN cells stained with an isotype matched negative control antibody.
3.4 *Extravillous trophoblast outgrowth expansion derives from proliferation of cytotrophoblasts in the tips of anchoring villi.*

The absence of Ki67 expression, and the contrasting high level of nuclear PCNA expression, in EVT outgrowths *in vitro* (section 3.4) caused confusion as to whether these cells were proliferating or not. In order to clarify this issue, villous explants which had
produced EVT outgrowths following 5 days of culture were fixed in paraformaldehyde, paraffin embedded, sectioned, and the expression of Ki67 and PCNA within villous tips from which outgrowths had been produced were examined.

In order to examine which cells in villi were currently committed to the cell cycle the expression of Ki67 and PCNA were examined in sections from 3 placentae fixed at the time of collection (t=0) and 37 sectioned explants from those placentae from that had produced outgrowth in culture. In placental tissue at t=0, Ki67 expression was evident in some villous cytotrophoblasts and some mesenchymal cells of all villi. The proportion of cytotrophoblast that expressed Ki67 varied largely between villi, with the majority of cytotrophoblasts in smaller villi expressing Ki67, whereas less than 50% of cytotrophoblasts expressed Ki67 in larger villi (Figure 3.5). In contrast, in first trimester placental tissue at t=0, PCNA was expressed in the cytotrophoblast layer of the vast majority of villi (Figure 3.6, panel A). Only occasional cells of the mesenchymal core expressed either Ki67 or PCNA (Figure 3.5; 3.6).

In villi which had been in explant culture for 5 days Ki67 expression was not observed in any mesenchymal cells, nor in the majority of cytotrophoblasts other than those in pockets of multilayered cytotrophoblasts in some villous tips (Figure 3.5). At this time PCNA was only expressed in some cytotrophoblasts in some villi, with fewer PCNA positive nuclei, and mesenchymal staining was extremely rare (Figure 3.6, panel B). PCNA expression was also observed in some syncytial knots being shed from the villi of placentae at t=0 (Fig 3.6, panel E).

At t=0, Ki67 expression was observed in the majority of multilayered cytotrophoblasts in villous tips from which EVT outgrowth was produced, and in proximal portions of EVT outgrowths, but Ki67 expression did not extend throughout the outgrowth (Figure 3.5). Strong PCNA expression at t=0 was observed in villous cytotrophoblasts at the base of trophoblast outgrowth but was reduced as EVTs migrated away from the placenta. Since EVT outgrowths were detached from the villous tips when explants were removed from cultures plates it was not possible to study Ki67 or PCNA expression in EVT outgrowths of villous explants other than in the most proximal EVTs. Ki67 expression was observed in only a small number of the multilayered cytotrophoblasts in the villous tips from which outgrowths were produced, and/or in a few EVTs proximal to the villous tip (Figure 3.5), while PCNA showed more frequent expression in these cells and
extended throughout most of the EVT\(^{s}\) detached from the culture plate. Ki67 and PCNA expression was also observed in multilayered cytotrophoblasts in tips from which EVT outgrowth was not evident in several explants.

Figure 3.5 – Photomicrographs of Ki67 expression in villous tissue at \(t=0\) (A, C) and in explants after 5 days in culture in 8% oxygen (B, D). Tips producing EVT outgrowth are shown (C, D). Staining of trophoblast layers was observed with the positive control antibody reactive with cytokeratin (E). No staining was observed with an irrelevant class matched negative control antibody (F).
Figure 3.6 – Photomicrographs of PCNA expression in villous tissue at \( t = 0 \) (A, C) and in explants after 5 days in culture in 8\% oxygen (B, D). Tips producing EVT outgrowth are shown (C, D). PCNA expression was also observed in syncytial knots (E). No staining was observed with an irrelevant class matched negative control antibody (F).
3.6 The effect of gestation on the production of extravillous trophoblast outgrowth from first trimester villous explants

In order to determine the effect of gestation on the frequency and size of EVT outgrowth, 3963 explants were cultured in either 8% or 1.5% oxygen. 1.5% or 8% oxygen was used instead of 20% oxygen in this work in order to provide conditions that mimic the placental environment in the first trimester in vivo more accurately. 13.8% (548) of explants floated in the culture media and, as they were not in contact with the Matrigel surface, these explants were excluded from further analysis. Overall, 18.6% (317/1702) of explants cultured in 8% oxygen, and 14% (239/1713) of explants in 1.5% oxygen produced EVT outgrowths. The percentage of explants from each placenta that produced EVT outgrowth declined progressively with increasing gestational age of the tissue, independent of oxygen concentration (F=0.012 and F=0.001 in 1.5% and 8% oxygen cultures respectively) (Figure 3.8).

Figure 3.8 - Scatterplot demonstrating the percentage of explants in 8% (♦) and 1.5% (●) oxygen conditions that produced EVT outgrowth in relation to gestational age. The percentage of explants from each placenta which produced EVT outgrowth in each culture is represented by one point on the graph. The percentage of explants that produce EVT outgrowth declined significantly with increasing gestational age in both 1.5% (F=0.012) and 8% (F<0.001) oxygen conditions.
3.7 Viability of cells in placental villous explants in culture

As placental villi contain a number of different cell types which each contribute to the function of the placenta and secrete different profiles of cytokines and growth factors that are known to play a role in trophoblast function and differentiation it was important to determine the viability of all cell types in the villi throughout the period of culture. In order to examine explant viability and cell death in detail several markers of cellular viability and apoptosis were examined in cultured explants.

3.7.1 Determination of cellular viability in villous explants during 5 days of culture using chloromethylfluorescin diacetate (CMFDA) and ethidium bromide.

In order to investigate the viability of trophoblasts in villous explants in culture, 726 explants from 9 placentae were cultured for up to 96 hours. 164 of these explants were stained with 5-chloromethylfluorescin diacetate (CMFDA) and ethidium bromide (EtBr), and visualized by confocal microscopy at 4 (n = 47), 24 (n = 17), 48 (n = 51) or 96 (n = 49) hours. After 4 hours in culture, the earliest timepoint measurable by this method, the viability of the syncytiotrophoblast layer was severely compromised as revealed by uptake of EtBr and failure to metabolize CMFDA (Figure 3.9, panel a). After 24 hours fragments of dead syncytiotrophoblast reminiscent of syncytial knots were observed apparently being extruded from the syncytiotrophoblast (Figure 3.9, panel b). After 48 and 96 hours of culture significant areas of viable syncytiotrophoblast were evident, but the villous cytotrophoblasts underlying the syncytiotrophoblast as well as the cells of the mesenchymal core were no longer viable (Figure 3.9, panels c and d).
Figure 3.9 - Optical sections of explants from a 12.2 week placenta stained with 5μM CMFDA and 2.5μM EtBr after 4 (a), 24 (b), 48 (c) and 96 (d) hours of culture and visualized by confocal microscopy. After 4 hours the syncytiotrophoblast was not viable as shown by the uptake of EtBr, however the cytotrophoblasts and cells of the mesenchymal core metabolised CMFDA and excluded EtBr (a). This non-viable syncytiotrophoblast layer was shed in syncytial knots as observed after 24 hours of culture (b). However, after 48 hours of culture the syncytiotrophoblast had regenerated and was able to metabolise CMFDA, whereas the cytotrophoblast and cells of the mesenchymal core were no longer viable (c). The pattern of staining seen after 48 hours of culture was repeated after 96 hours of culture (d).
3.7.2 Determination of cellular viability in villous explants during the first 5 hours of culture using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)

In order to confirm the rapid syncytiotrophoblast degeneration in villous explants observed by CMFDA and EtBr staining (3.7.1), the MTT assay was used to assess cellular viability between 0 and 5 hours in culture (n = 45 explants). After one hour in culture (n = 9) all cell types in the villi were viable (Figure 3.10, panel A). The MTT staining in the villous cytotrophoblast was often more intense than that in the syncytiotrophoblast or cells of the mesenchymal core (Figure 3.10, panel A). From 4 hours of culture (n = 18) approximately half of the syncytiotrophoblast was non-viable whereas, the cytotrophoblast and cells of the mesenchymal core remained viable (Figure 3.10, panel B).

![Figure 3.10 - A) Photomicrograph showing MTT staining of a villous from an explant of 8.4 weeks gestation 1 hour after collection. MTT staining (purple) was present in the syncytiotrophoblast, cytotrophoblast and cells of the mesenchymal core. MTT staining in the cytotrophoblast layer (short arrow) appeared denser than that of the syncytiotrophoblast (long arrow). B) Photomicrograph showing MTT staining of a villous from an explant of 8.4 weeks gestation 4 hours after collection. The syncytiotrophoblast (long arrow) was no longer viable, as shown by an absence of MTT metabolism. The cytotrophoblast (short arrow) and mesenchymal cells (M) remain viable](image-url)
3.7.4 Examination of apoptosis in villous explants by DNA laddering

In order to examine whether the loss of cellular viability in cultured villous explants was due to apoptotic death, explanted villi from each of the 9 placentae were pooled and DNA was extracted and examined for DNA laddering by agarose gel electrophoresis after 0, 48 and 96 hours of culture. DNA laddering was virtually absent from freshly harvested villous tissue, with a faint ladder present in the DNA from only 1 of 9 placentae. DNA laddering was observed in 6 of 9 placentae at both 48 and 96 hours of culture (Figure 3.11).

![Figure 3.11 – Representative gel showing DNA laddering in extracts from placentae of 12.2 weeks (A, B, C) or 12.0 weeks (D, E) of gestation. DNA extracted from fresh tissue (A) or after 48hrs (B, D) or 96hrs (C, E) in explant culture, was run on a 2% (w/v) agarose gel and visualized after staining the gel with Ethidium Bromide. A control sample of U937 cells that had been induced to undergo apoptosis by treatment with camptothecin was used as a positive control of DNA laddering (right hand lane). In addition, a 123bp marker was run to indicate that the DNA fragments producing the laddering pattern were of the expected size. Bands of DNA laddering are indicated by the red arrows.](image-url)
3.7.5 Determination of apoptotic cell death in cultured villous explants by activated caspase-3 and cytokeratin neo-epitope (M30) expression

In order to determine which cells in the villous explants were apoptotic, the expression of activated caspase-3 and the M30 cytokeratin neo-epitope were examined in 24 explants of 8 weeks of gestation by immunohistochemistry. In freshly harvested villous tissue only rare cells stained positive for activated caspase-3 (Figure 3.12) and no staining was observed with the cytokeratin neo-epitope M30 antibody (Figure 3.13). The activated caspase-3 cells were primarily confined to the mesenchymal core of the villi, suggesting that apoptosis was not occurring in the syncytiotrophoblast of this fresh tissue (Figure 3.12). However, after 48 (n = 12 explants) and 96 (n = 12 explants) hours of culture activated caspase-3 and M30 cytokeratin neo-epitope staining was evident in occasional cytotrophoblasts and small stretches of syncytiotrophoblast (Figure 3.12, 3.13). Increased expression of activated caspase-3 in the cells of the mesenchymal core was seen from 48 hours of culture, although the levels of expression in these cells varied greatly between individual explants (Figure 3.12). Overall, there was no one cell type in which activated caspase-3 or M30 cytokeratin neo-epitope staining was predominant.
Figure 3.12 - Photomicrographs showing immunolocalisation of activated caspase-3 in fresh tissue (a), and explants after 48 (b) and 96 (c) hours of culture from an 8.5wk placenta. Negative controls incubated with an irrelevant class matched control antibody showed no staining (d). Positive controls showed cytokeratin expression in the trophoblast layers (e).
Figure 3.13 - Photomicrographs showing immunolocalisation of M30 cytokeratin neoepitope in fresh tissue (a), and explants after 48 (b) and 96 (c) hours of culture from an 8.5wk placenta. Negative controls incubated with an irrelevant class matched control antibody showed no staining (d). Positive controls showed cytokeratin expression in the trophoblast layers (e). Staining with the cytokeratin neoepitope M30 cytodeath was less extensive than that observed for activated caspase 3 (Figure 3.12).
3.7.6 The effect of oxygen on cell viability in first trimester villous explants

In order to determine whether oxygen affected the viability of cells in villous explants 103 explants from 4 placentae of 7 to 9 weeks of gestation were cultured in either 1.5% (n=37), 8% (n=39) or 20% (n=27) oxygen, stained with CMFDA and EtBr, and visualized by confocal microscopy after 48 (n =54) or 96 (n =49) hours in culture. There was no discernable difference between the viability of explants cultured in 1.5%, 8% or 20% oxygen (Figure 3.14). In section 3.7.1 explants cultured in 20% oxygen showed rapid death of the syncytiotrophoblast, which was regenerated by 48hrs, by which time the vast majority of the cytotrophoblasts and cells of the mesenchymal core had died. In this work, the syncytiotrophoblast was not viable at either 48 or 96 hours as shown by the uptake of EtBr and the absence of CMFDA metabolism in all oxygen conditions (Figure 3.14). However, in comparison to the pattern of cell death of explants in section 3.7.1, the time frame of cytotrophoblast and mesenchymal cell death was retarded by several days (see section 4.5), and both of these cell types remained viable at 48 and 96 hours in all oxygen conditions, although a degree of inter-villous variability in the level of mesenchymal cell viability was observed within explants in all oxygen conditions at 96 hours (Figure 3.14). However, by 10 days of culture all cells in the villi were dead apart from those cytotrophoblasts in multilayered cell islands in villous tips (Section 4.5, Figure 4.4).
Figure 3.14 – Optical sections of villous explants of 8 or 9 weeks gestation stained with 5μM CMFDA and 2.5μM EtBr after 48 (A, C, E) or 96 (B, D, F) hours of culture in 1.5% (A, B), 8% (C, D) or 20% (E, F) oxygen and visualized by confocal microscopy.
3.7.7 Summary of results examining the viability of cells in villous explants

The vast majority of the syncytiotrophoblast had reduced viability after 4 hours in culture and large areas of the dead syncytium were shed by 24hrs in culture as syncytial ‘knots’. The syncytiotrophoblast layer was partially regenerated as the culture progressed beyond 24 hours, whereas the underlying cytotrophoblast layer and cells of the mesenchymal core were largely non-viable from 48 hours of culture onwards. Syncytiotrophoblast regeneration did not completely deplete the population of underlying cytotrophoblasts, as a non-viable cytotrophoblast layer remained present at later time points, although this layer was discontinuous in places. Although there was sporadic apoptotic death of all cell types in the explants, by and large trophoblast death appeared to be primarily non-apoptotic. Explant viability was not altered by culture in 1.5% or 8% oxygen.

3.8 Summary of key findings

This chapter has described the characterization of a two dimensional model of EVT outgrowth from first trimester villous explants. This work, in particular the investigation of explant viability in relation to EVT outgrowth production, has resulted in the identification of a group of cytotrophoblasts in the anchoring villous tips from which EVT outgrowth is produced. These cytotrophoblasts display unique properties, leading to the hypothesis that they constitute a separate villous cytotrophoblast sub-population.

The key findings were:

- Outgrowths from first trimester villi explanted onto Matrigel were confirmed to be comprised solely of trophoblasts by morphology and cytokeratin immunohistochemistry.
- EVT outgrowths expressed the MHC class I protein HLA-G characteristic of EVT outgrowths in vivo.
- EVT in outgrowths visible on the Matrigel surface were not actively proliferating.
- Ki67 and PCNA are expressed in a substantial proportion of cytotrophoblasts in villi at t=0, but expression is greatly reduced in cytotrophoblasts of both anchoring and floating villi in explants cultured for 5 days in 8% oxygen.
- EVT outgrowths appear to expand by proliferation of cytotrophoblasts in the tips of anchoring villi and the regions of cell columns proximal to the villous in vivo and in cultured explants.
• The percentage of explants which produce EVT outgrowth declines as gestational age increases from 8 to 12 weeks of gestation.

• The syncytiotrophoblast of first trimester explants showed significantly reduced viability within 4 hours of culture, but regenerated by 48 hours of culture.

• The vast majority of villous cytotrophoblasts and cells of the mesenchymal core were not viable from 48 hours of culture. However, multilayered cytotrophoblasts in villous tips from which EVT outgrowth was produced were observed to remain viable for extended culture periods.

• Activated caspase-3 and cytokeratin neo-epitope M30 immunohistochemistry show that, while sporadic apoptotic cell death occurred in all cell types in the explants, neither the initial syncytiotrophoblast death, nor the large scale of cytotrophoblast and mesenchymal cell death from 48 hours of culture, could be attributed to apoptosis.
3.9 Discussion

3.9.1 A two-dimensional model of extravillous trophoblast outgrowth from first trimester villous explants.

In this chapter I have described a first trimester explant model from which EVT outgrowth was produced. The explants in this model were cultured on an extremely thin layer of Matrigel such that the EVT outgrowth extended across the Matrigel surface in a two-dimensional sheet. This two-dimensional outgrowth is a feature unique to this explant model, and has several advantages. Firstly, EVT outgrowth was easily detectable and its expansion was easily visualized by phase contrast microscopy, thus allowing the frequency and area (as will be described in chapter 5) of the outgrowth to be readily quantified. Secondly, Matrigel is known to contain a number of growth factors and cytokines which are not accurately defined and which vary between batches. As trophoblast differentiation, and in particular EVT outgrowth, is regulated by a number of interacting cytokines, using an extremely thin layer of Matrigel reduces the levels of unknown cytokines and growth factors in the culture. Therefore, by minimizing the amount of Matrigel the potentially confounding effects of cytokine variation between batches is reduced. Thirdly, it is possible to examine the expression of proteins by EVTs using immunohistochemistry, allowing characterization of the outgrowth in situ.

In this work EVT outgrowth was easily identified by its distinctive morphology, and was easily distinguishable from fibroblast outgrowths, which occurred only rarely in this model. Cytokeratin-7 positive (and vimentin negative) EVTs appeared on the Matrigel surface as broad cells, usually of a rounded rectangular shape, with distinctive dark peri-nuclear granules. Occasional cell processes extended out from the EVTs, particularly in those cells which are at the distal edge of the outgrowth or those that have broken off from the sheet of EVT outgrowth. EVTs were also often seen growing out from villous tips in columns similar to those observed in vivo. In vivo, EVTs express the Class Ib MHC molecule HLA-G, which is able to actively inhibit cytotoxic T-lymphocytes and natural killer cell activity (Hofmeister & Weiss, 2003). Immunohistochemistry with HLA-G further confirmed that these cells were EVTs. Determination of MHC-I expression by immunohistochemistry using the antibody W6/32 confirmed the accuracy and distribution of HLA-G expression over the entire outgrowth. Taken together, these
results provide convincing evidence that the outgrowths produced in this model contained pure EVTs, and that they could be readily identified by phase contrast microscopy.

3.9.2 The frequency of extravillous trophoblast outgrowth

It has previously been shown that in this model approximately 25% of explants produce EVT outgrowth when cultured in 20% oxygen (James, 2001). In this work explants cultured in 20% oxygen showed an almost identical overall rate of outgrowth of 24.7%. This contrasts with other models in which the frequency of explants that produced EVT outgrowth is reported to be greater than 70% (Genbacev et al., 1993; Irving et al., 1995; Matalon et al., 2005; Newby et al., 2005). The exception is Irving and colleagues who reported 39% of explants producing EVT outgrowth (Irving et al., 1995). However, many studies using explant cultures do not state the percentage of explants that produced EVT outgrowth (Aplin et al., 1999; Caniggia et al., 2000; MacPhee et al., 2001; Nishimura et al., 2004; Vivovac et al., 1995). It is possible that the thinness of the layer of Matrigel used in this work adversely affected the frequency of outgrowth in comparison to three-dimensional models in which the villous is embedded in the Matrigel. Alternatively, explants used in the model in this work were smaller (on average 8 mg wet weight) than those used by most researchers and therefore it is possible that the lower frequency of outgrowth may be a result of each explant containing only a few villous tips from which outgrowth could be produced. However, the most likely reason that a lower percentage of explants produced EVT outgrowth in this model is due to the random selection of large numbers of villi, providing a greater representation of villi in the placenta as a whole in this work whereas, in most other studies only anchoring villi are selected for explanting.

3.12.3 Proliferation of extravillous trophoblast outgrowths

The expansion of EVT outgrowth in first trimester explant culture models has been reported to derive from the proliferation of Ki67 positive EVTs proximal to villous tips (Aplin et al., 1999; Caniggia et al., 1997a; Genbacev et al., 1992; Nishimura et al., 2004). Immunostaining fresh first trimester placentae with antibodies reactive with PCNA and Ki67 confirmed, as previously reported, that the staining pattern is similar to the pattern in vivo. However, in this work it was also shown that EVTs in outgrowths visible on the
Matrigel surface do not express Ki67, and are very unlikely to be actively proliferating. However, the most proximal EVTs may have been concealed by the villus, and thus it would not be possible to visualize these cells. In agreement with previous studies (Aplin et. al., 1999; Genbacev et. al., 1993), a further marker of proliferation, PCNA, was expressed throughout EVT outgrowths. The greater number of EVTs expressing PCNA than Ki67 in this work may be explained by the different half-lives of the Ki67 and PCNA antigens. Ki67 is an antigen expressed in the granular components of the nucleolus during late G1, S, G2 and M phases of cell division. Since Ki67 has a half life of less than one hour it is only expressed in cells that are actively undergoing cell division (Gerdes et. al., 1984; Schraper et. al., 1987). PCNA is also expressed selectively during the cell cycle, being elevated in the nucleus during late G1 phase, immediately before the onset of DNA synthesis, then achieving maximal levels during S-phase with levels declining during G2 and M phases (Takasaki et. al., 1981). However, PCNA has a long half life, the exact length of which has not been determined but exceeds 20hrs, and therefore it is expressed in the nuclei of cells that are no longer actively dividing, particularly in cells that proceed rapidly through the cell cycle (Allegranza et. al., 1991; Korgun et. al., 2006; Scott et. al., 1991). In addition, PCNA is involved in polymerase-mediated DNA repair so may be expressed in non-proliferating cells, and deregulation of PCNA expression in some forms of neoplasia is observed such that the relationship between PCNA and cell proliferation is lost (Celis & Madsen, 1986; Hall et. al., 1990; Shivji et. al., 1992; Toschi & Bravo, 1988). Therefore, PCNA is not as reliable a marker of proliferation as Ki67 (Allegranza et. al., 1991; Scott et. al., 1991). In this work, EVT outgrowths were examined by immunohistochemistry after 3 days in culture and as outgrowths are not formed immediately this short period of culture means that the majority of outgrowths would have been formed in the last 48 hours of culture. Thus, as the EVTs present in outgrowths were derived from proliferating cytotrophoblast cells, a large number of EVTs in the outgrowth will have been part of a proliferative cycle within the half-life of the PCNA antigen, and would express PCNA even though they may not be accurately proliferating in the outgrowth. The contrasting results between Ki67 and PCNA expression demonstrate how PCNA results must be carefully examined in context before conclusions about the implications of cellular PCNA expression can be drawn.

Ki67 staining demonstrated that multilayered cytotrophoblasts in villous tips associated with EVT outgrowth were proliferating at the end of the culture period. In addition,
some proliferative trophoblasts were observed in the EVTs immediately adjacent to these cell islands. These EVTs in the outgrowth may not have been visible on the Matrigel surface when explants were stained in situ due to the explant blocking the field of view. This observation explains how EVT outgrowths are able to continue to expand in culture when those EVTs visible in outgrowths on the Matrigel surface were not proliferating and is in agreement with the staining pattern for proliferation seen in vivo (Korgun et al., 2006; Wolf & Michalopoulos, 1992). The extent of cytotrophoblast and EVT proliferation in explants after 3 days in culture was substantially less than that observed in freshly harvested anchoring villi (t = 0). This may be due to a decline in the number of viable villous cytotrophoblast in these tips, or a result of the culture model not providing the exact repertoire of cytokines and growth factors these cells would be likely to be exposed to in vivo.

In summary, these data show that EVT outgrowth expansion in the explant model used in this work is driven by the proliferation of both multilayered cytotrophoblasts in the tips of anchoring villi, and EVT in regions of the cell columns most proximal to the villi, as occurs in vivo. Despite differences in the extent of proliferation between villous tissue at the time of collection, and explants after 5 days in culture, explants retain the ability to produce EVT outgrowths due to continued, albeit decreased, proliferation of cytotrophoblasts in cell islands of anchoring villi. Thus, this model is a useful for the study of EVT differentiation.

3.12.4 Syncytiotrophoblast turnover

In thin sections of freshly harvested first trimester placental tissue, occasional nuclei in the syncytiotrophoblast expressed PCNA. However, Ki67 expression was not seen in any syncytiotrophoblast nuclei. The syncytiotrophoblast is formed by fusion from the underlying cytotrophoblast layer and does not proliferate (Boyd & Hamilton, 1970). However, due to the long half life of PCNA discussed above, the PCNA expression observed in nuclei in this layer may remain as an artifact from cytotrophoblast proliferation prior to fusion. Indeed, PCNA positive nuclei have been previously reported in the syncytiotrophoblast (Korgun et al., 2006). It was extremely interesting to observe occasional PCNA expression in nuclei in syncytial knots being shed from the villi (Figure 3.6, panel E). The process of cytotrophoblast proliferation and fusion has
been reported to take several days, whereas the process from fusion to extrusion of nuclei in syncytial knots has been estimated to take several weeks (Benirschke & Kaufmann, 2000; Huppertz et al., 1998). However, these calculations are based on rough assumptions incorporating the number of proliferating cytotrophoblasts, the weight gain of the syncytiotrophoblast during pregnancy and the extrusion rate of apoptotic nuclei (Benirschke & Kaufmann, 2000; Huppertz et al., 1998). The results in this thesis show remarkably different findings as, for PCNA to be expressed in nuclei in extruding syncytial knots, at least some nuclei must move reasonably rapidly from the villous cytotrophoblasts into extruded knots.

3.12.6 The effect of gestation on extravillous trophoblast outgrowth

The effect of gestational age on EVT outgrowth from villous explants has not previously been reported. In this work, independent of oxygen concentration, the percentage of randomly selected explants that produced EVT outgrowth declined progressively with the increasing gestational age of the tissue. It is well known that the ratio of floating to anchoring villi increases as gestation progresses, particularly in the 2nd and 3rd trimesters, but this work shows that even during the last five weeks of the first trimester the gestational age of placentae is important with respect to EVT outgrowth production. It is widely believed that all villous cytotrophoblasts from first trimester placentae are bipotent and can form either syncytiotrophoblast or EVTs. In line with this belief it has been suggested recently that all villi in the first trimester placenta are capable of forming into anchoring villi and producing EVTs (Baczyk et al., 2005). The results in this work using very large numbers of explants argue against that suggestion and demonstrate that not all villi have the potential to become anchoring villi, but rather that the numbers of villi with the potential to develop into anchoring villi decrease as the placenta expands with increasing gestational age. The observed effect of gestational age on EVT outgrowth production also demonstrates that there are significant differences in trophoblasts within the first trimester, and indicates that care must be taken to not assume all first trimester tissue samples from varying gestations will exhibit identical behavior.
3.12.7 The viability of cells in villous explants in culture

The viability of explants in culture has significant potential to affect the results obtained from explant models, particularly in the study of trophoblast differentiation. Therefore, when establishing this explant model it was necessary to address previous concerns raised from reports that the syncytiotrophoblast rapidly degenerates in explant cultures (Palmer et al., 1997; Siman et al., 2001; Watson et al., 1995; Watson et al., 1998). Palmer and colleagues studied cultured first trimester chorionic villi by electron microscopy, and demonstrated degeneration of the syncytiotrophoblast by 24 hours of culture (Palmer et al., 1997). A new syncytiotrophoblast layer was then formed from the viable underlying cytотrophoblasts by 48 hours in culture, and was maintained until at least 120 hours of culture (Palmer et al., 1997). In this work that finding was confirmed using CMFDA, a cell-permeable dye which is metabolized in viable cells to a cell-impermeant fluorescent green dye, and EtBr, which is only able to enter cells when membrane integrity has been compromised indicating cell death. The exclusion of EtBr and metabolism of CMFDA provide two independent and objective measures by which viability can be assessed simultaneously in individual cells. Using these markers it has been shown that the vast majority of the syncytiotrophoblast is non-viable after 4 hours in culture and that large areas of the dead syncytium are shed by 24 hours in culture. It seems likely that, despite very gentle treatment of the tissues, the initial massive syncytiotrophoblast death was triggered in the early stages of culture or during the preparation of the tissue. The syncytiotrophoblast also rapidly lost the ability to metabolize MTT after the placentae were harvested, but in contrast to the uptake of EtBr and lack of CMFDA metabolism over the entire syncytiotrophoblast after 4 hours in culture, significant areas of syncytiotrophoblast retained the ability to metabolize MTT at this timepoint. However, care must be taken in using MTT as an indicator of cellular viability as the mechanism of cellular MTT reduction is not completely understood (Liu et al., 1997). MTT is generally accepted to be reduced by the mitochondrial electron transport chain, based on a study of cell homogenates by Slater et al. (1963) (Slater et al., 1963). However, in intact cells MTT is also able to be reduced by NADH- and NAD(P)H-dependent mechanisms in intracellular vesicles, and is therefore able to be affected by factors such as oxidative stress (Liu et al., 1997). Exposure of early first trimester villous tissue, which exists in a hypoxic environment in vivo and does not contain protective antioxidant enzymes, to atmospheric oxygen concentrations would increase cellular levels of reactive oxygen species (Watson et al., 1997). High levels of reactive oxygen species have been

In this work, after 24 hours in culture the formation of syncytial ‘knots’ that are involved in the process of syncytiotrophoblast shedding from the villi was observed. The extrusion of terminally differentiated syncytium through the formation of syncytial ‘knots’ is essential for the renewal of the syncytiotrophoblast and the growth of the placenta (Hempstock et. al., 2003; Huppertz et. al., 1998; Huppertz et. al., 1999). The syncytiotrophoblast layer was partially regenerated as cultures progressed beyond 24 hours, whereas the underlying cytotrophoblast layer was largely non-viable. This work led to the proposal that the regenerated syncytiotrophoblast was formed from the cytotrophoblasts that were viable during the first 1-2 days of culture. The syncytiotrophoblast regeneration did not completely deplete the population of underlying cytotrophoblasts, as a non-viable cytotrophoblast layer remained present at later time points, although this layer was discontinuous in places. It is not clear why these cytotrophoblasts and the cells of the mesenchymal core died with prolonged culture, but possibly a lack of specific growth factors from the damaged syncytiotrophoblast, or the loss of factors contributed by the fetal circulation contributed to the death of these cells.

It is also important to note that the structure and cellular constituents of the mesenchymal core change as villi mature and become more cellular with advancing gestation. Thus, the anchoring villi that this study concentrated on may not be entirely representative of large villi such as stem villi. The death of cells in the mesenchymal core is also supported by the rarity of fibroblast outgrowths in this model.

Since massive cell death has occurred in these cultures the question may be asked “Why do the explants remain intact?”. In the sterile culture system in this work there is no bacterial breakdown of the tissues. In vivo, cells of the immune system, particularly macrophages, would be responsible for removing dead cells, but in this in vitro system there are only low levels of macrophages contained in the villous core and consequently the dead but sterile tissue might be expected to remain intact for prolonged periods of time as has been observed.

The rapid degeneration of the syncytiotrophoblast in this model raises some concerns for the use of explant models in the study of syncytiotrophoblast function. In such studies
researchers should be aware of the rapid degeneration of the syncytiotrophoblast and design experiments taking this into account. However, as the observed cell death does not affect the ability of explants to produce EVT outgrowth, and viable trophoblast outgrowth is able to be produced for up to three weeks in culture, the explant model remains a good method for the study of trophoblast invasion.

It is unclear why the exact pattern of cell viability in villous explants consistently observed in 164 explants in the initial work in section 3.7.1 was not evident in the 103 explants investigated in section 3.7.6 to determine the effect of oxygen on cell viability. Results in both experiments were consistent within the experiments, and a large number of explants were used in each case. The death of the syncytiotrophoblast early in culture was consistently observed in all experiments. However, it appears the extent of cytotrophoblast and mesenchymal cell death observed within a few days in section 3.7.1 may have been retarded somewhat in the later experiments in 3.7.6. As the different experiments were undertaken several years apart, it is possible that factors present in a particular batch of FBS or Matrigel used may have altered the rate of death of the explants in either case. In addition our laboratory was relocated between these experiments, potentially resulting in changes in the quality of water supply, the equipment used for cell culture and other confounding factors. The differences in viability observed between these two experiments do not take away from the major finding that a large scale of villous cell death is possible within a relatively short time frame, and that this possibility must be taken into consideration in the future work involving villous explants. Furthermore, the contrasting results further illustrate that caution must be taken to carefully characterise explant viability when it is an important factor in interpreting the experimental results. The contrasting patterns of viability between sections 3.7.1 and 3.7.6 also do not detract from the finding that oxygen concentration did not alter the viability of explants over 96 hours of culture.

3.12.7.1 Cell death in cultured explants is not primarily via apoptosis

The rare staining of the syncytiotrophoblast with antibodies to the cytokeratin neo-epitope M30 and activated caspase-3 as well as the absence of DNA laddering suggest necrotic (or some other non-caspase mediated mechanism of death) rather than apoptotic death of the syncytiotrophoblast, particularly during the first 24 hours of
culture. Apoptotic cell death does occur during the later stages of explant culture, but staining for activated caspase-3 and cytokeratin neo-epitope M30 indicates that this arises from low level sporadic apoptotic death of all villous cell types and that much of the cell death that occurs during the later stages of culture is non-apoptotic. These results are in contrast to previous findings of high levels of trophoblast apoptosis within 24 hours in term villous explants also detected using the cytokeratin neo-epitope M30 antibody (Di Santo et. al., 2003). However, it is possible that such differences in the level of observed apoptosis represent intrinsic differences between placentae in the first trimester studied in this work and at term as studied by Di Santo et. al. (Di Santo et. al., 2003). It may also be possible that programmed cell death pathways not involving the caspases have been activated in the syncytiotrophoblast leading to the larger scale degeneration of this layer in the first hours of explant culture.

3.12.9 Summary

This data presented in this chapter has described the characterization of a first trimester explant model which, due to its random selection of large numbers of villi, provides a representative sample of villi from the entire placenta. The EVT outgrowth produced in this model is phenotypically similar to EVT outgrowth observed in vivo. The proportion of explanted villi that produced EVT outgrowth declined with advancing gestational age in the first trimester, suggesting that not all villi have the potential to become anchoring villi. Investigation of the viability of villous explants in culture confirmed previous reports that the syncytiotrophoblast dies rapidly by a non-apoptotic mechanism when placed in explant culture. Cultures using a large number of explants showed that the syncytiotrophoblast was partly regenerated within 48 hours of culture, whereas the majority of villous cytotrophoblasts underlying the syncytiotrophoblast and cells of the mesenchymal core die as soon as the first week of culture, although it appears that the rate at which this mass cell death occurs can be retarded by potential variations in culture conditions. Finally, the explant model described will be a useful tool by which to study the process of EVT differentiation in vitro.
Chapter Four:

The Identification, Isolation and Characterisation of ‘Extravillous Trophoblast Progenitors’
4.1 Introduction and rationale

It is a widely held belief that villous cytotrophoblasts are a bipotent progenitor population capable of differentiating into either EVTs or syncytiotrophoblast. However, it remains unclear which factors are essential for cytotrophoblast differentiation into either of these lineages. This chapter describes the identification of a population of ‘EVT progenitors’ in the tips of villi from which EVT outgrowth is produced that exhibit different characteristics to the cytotrophoblasts which underlie the syncytiotrophoblast around the majority of each villous. This identification led to the formation of the hypothesis that ‘EVT progenitors’ constitute a separate population of cytotrophoblasts committed to differentiation into EVTs from 8 weeks of gestation. In order to further study this population of EVT ‘progenitors’ in more detail I have isolated viable trophoblasts based on their ability to survive for extended periods in explant culture and studied their characteristics in vitro. This population of isolated trophoblasts will be referred to as putative EVT progenitors.

4.2 Identification of viable cells in villous tips from which extravillous trophoblast outgrowth is produced.

Of the villous explants used to determine cellular viability in the explants in Chapter 3 (section 3.7.1), 19.1% (55) of the 288 explants cultured from 9 placentae of 8 to 12 weeks of gestation produced areas of EVT outgrowth by 96 hours of culture. A comparison using CMFDA and EtBr staining of those explants that produced EVT outgrowth (n = 39) with those that did not produce outgrowth (n = 62) after 48 or 96 hours showed no difference in the viability of either the syncytiotrophoblast or villous mesenchymal core. EVT outgrowth became macroscopically obvious by 96 hours in culture despite the death of most of the villous cytotrophoblasts and cells of the villous mesenchymal core (as shown in section 3.7.1) and the EVT outgrowth continued to expand until it was limited by the destruction of the thin layer of Matrigel used in the culture system. After 96 hours of culture the only villous cytotrophoblast population that remained viable were the pockets of multilayered cytotrophoblast that were located in villous tips directly behind the EVT columns (Figure 4.1).
Figure 4.1 - Optical section of a villous tip from an 8.5 week explant that produced EVT outgrowth, stained with 5μM CMFDA and 2.5μM EtBr and visualized by confocal microscopy after 48 hours in culture. This tip is believed to be a site of trophoblast outgrowth where the cytotrophoblasts (arrow) have remained viable despite a loss of viability in all cells of the surrounding villi. The cytotrophoblasts have breached the syncytiotrophoblast layer to produce EVT outgrowth of which only the first few cells can be seen in this image.

4.3 EVT outgrowth in 3 sequential week-long passage cultures

In order to examine whether EVT outgrowth would continue for extended periods, 376 villous explants from 4 placentae (two each of 9 and 11 weeks of gestation) were cultured for 7 days (primary culture) then transferred to fresh Matrigel-coated culture plates. The culture was continued for a further 7 days (secondary culture) and the explants again transferred to fresh plates for an additional 7 days of culture (tertiary culture). In these experiments 32.6%, 8.0% and 3.2% of the explants produced EVT outgrowth in the primary, secondary and tertiary cultures, respectively (Figure 4.2). Interestingly, 25% of explants that produced trophoblast outgrowth in the second passage did not produce EVT outgrowth in the first passage.
4.4 EVT outgrowth is able to continue over sequential passage cultures due to the survival of cytotrophoblasts in cell islands in anchoring villous tips

In order to confirm the extended viability of cytotrophoblasts in villous tips as shown in section 4.2, explants at the end of the second passage culture were left *in situ*, stained with CMFDA and EtBr, and examined by inverted confocal microscopy. As previously shown (section 4.2) the majority of cells in the villi were not viable. However, the cytotrophoblasts in the villous tips from which outgrowth was produced, and the outgrowth itself, remained viable (Figure 4.3).
Figure 4.3 - a) An optical section observed by confocal microscopy through a villous tip from an 8.2wk placental villous explant that produced EVT outgrowth, and was then transferred into a fresh culture well. After one week in the second passage culture the explant was stained with CMFDA and EtBr. The arrow indicates viable cells in the tip from which EVT outgrowth was produced, whereas the majority of other cells in the villus are no longer viable. b) Transmitted-light photomicrograph of the same field shown in (a) demonstrating the morphology of the villous tip and EVT outgrowth.
4.5 Isolation of putative EVT progenitors from explants

In order to isolate the putative EVT progenitors that were shown to remain viable following extended explant culture, explanted villi that had been cultured in a flask for 10 days were subjected to sequential trypsin digestion. 12 explants from 4 placentae were removed from culture prior to trypsin digestion, stained with CMFDA and EtBr, and examined by confocal microscopy to confirm that the cytotrophoblasts located in the multilayered cell islands in anchoring villous tips were the only viable cells in these explants (Figure 4.4). The cells obtained from the 2nd to 5th sequential trypsin digestes were pooled and cultured on a thin layer of Matrigel. After 24hrs, cellular debris was washed away with PBS, leaving a population of viable cells attached to the Matrigel which will henceforth be referred to as putative viable cells (Figure 4.5). The vast majority of these putative EVT progenitors showed the distinctive morphology of trophoblasts, with a rounded rectangular shape, peri-nuclear granules and large nuclei. Over 4 days in culture the number of these cells increased, and small groups of cells became evident (Figure 4.5).
Figure 4.4 – Optical section obtained by confocal microscopy of a villous from an 8.1wk explant stained with 5μM CMFDA and 2.5μM EtBr and visualized by confocal microscopy after 10 days in culture. CMFDA positive cells are present in the villous tip only (arrow).
4.6 Determination of putative EVT progenitor purity

In order to determine the purity of isolated putative EVT progenitors the cells isolated from 3 placentae were assessed by immunohistochemistry using antibodies against cytokeratin-7 (to label the trophoblasts), and vimentin (to label any contaminating cells). The percentage of cytokeratin or vimentin positive cells in 5 random fields at 10x magnification were counted over the first 4 days of culture. On average the population of cells obtained were >90% trophoblasts on day 1 of culture (Figure 4.6, 4.7). However, as the cultures progressed the rapid proliferation of the small number of contaminating cells resulted in a decline in trophoblast purity (Figure 4.7).
Figure 4.6 – Photomicrographs of *in situ* immunohistochemistry showing that the putative EVT progenitors were trophoblasts. Putative EVT progenitors stained with cytokeratin (A) Some vimentin positive cells were also evident (B, arrow). Sections of first trimester villous tissue were stained with cytokeratin (C) or vimentin (D) as true controls. No staining of putative EVT progenitors was observed with an irrelevant class matched control antibody (E).
Figure 4.7 – Putative EVT progenitor purity as determined by immunohistochemistry with cytokeratin or vimentin from day 1 to 4 of culture. The number of cells stained with antibodies reactive with either cytokeratin or vimentin, and the number of unstained cells (shown by nuclear haematoxylin staining) were recorded in each well.

An overlap in the percentage of vimentin positive and cytokeratin positive cells was observed, as there was an average difference of 10.59% (±2.2%) in the percentage of non-trophoblasts in culture when calculated by counting those which did not stain with cytokeratin versus calculated by counting those which did stain with vimentin (Figure 4.7). In order to clarify the source of this overlap, immunohistochemistry using fluorescent double-labelling with antibodies reactive with cytokeratin 7 and vimentin was performed. This determined that no cells were positive for both cytokeratin and vimentin.

### 4.7 Expression of αvβ6 integrin by putative EVT progenitors

In the placenta αvβ6 integrin has been reported to be expressed only in those cytotrophoblasts which form multilayered cell islands in anchoring villous tips (Zhou et al., 1997). In order to determine if the isolated putative EVT progenitors were the EVT progenitors present in the tips of anchoring villi, cultures of isolated putative EVT
progenitors were stained in situ with antibodies reactive with αvβ6 integrin. αvβ6 integrin was expressed in all cells with trophoblast morphology (Figure 4.8). In order to confirm that αvβ6 integrin was not expressed by EVTs, and that the putative EVT progenitors were not differentiated EVTs, EVT outgrowths from villous explants were also stained with anti-αvβ6 integrin in situ. No expression of αvβ6 integrin was seen in EVTs (Figure 4.8). Sections of fresh-frozen first trimester placenta used as positive controls showed that αvβ6 integrin expression was not present in floating villi, but was seen in the tips of some anchoring villi which had produced EVT outgrowth (Figure 4.8).
Figure 4.8 – Photomicrographs showing putative EVT progenitors stained with antibodies reactive with αvβ6 integrin (A, B), cytokeratin (C) and vimentin (D). EVT outgrowths were also probed with antibodies reactive with αvβ6 integrin (E). Fresh frozen first trimester placental sections were used as a positive control for αvβ6 integrin expression (F). Cells were counterstained with haematoxylin. Images A and F was developed with AEC, whereas all other images were developed using DAB.
4.8 Lack of expression of tenasin by isolated putative EVT progenitors

In the placenta tenasin expression has been reported sub-adjacent to the multilayered cell islands in anchoring villous tips \textit{in vivo} (Castellucci \textit{et. al.}, 1991). In order to examine whether putative EVT progenitors were the source of tenasin expression in anchoring villi, putative EVT progenitors were stained \textit{in situ} with antibodies reactive with tenasin. No expression of tenasin by putative EVT progenitors was observed (Figure 4.9). Furthermore, tenasin was not produced by EVT outgrowths from explants \textit{in vitro} (Figure 4.9). Sections of fresh frozen first trimester placenta used as positive controls showed that tenasin expression was not present in floating villi, but was seen in the tips of some anchoring villi immediately adjacent to cell islands of multilayered cytotrophoblasts, or at the base of EVT outgrowths (Figure 4.9).
Figure 4.9 – Photomicrographs of putative EVT progenitors stained with antibodies reactive with A) tenascin, B) vimentin or C) cytokeratin. EVT outgrowths were also stained with antibodies reactive with tenascin (D). Fresh frozen first trimester placental sections were used as a positive control for tenascin expression (E). Cells were counterstained with haematoxylin.
4.9 Proliferation of putative EVT progenitors in culture

A key characteristic of cytotrophoblasts in the multilayered cell islands of anchoring villous tips is their ability to proliferate, as shown by their expression of Ki67 (Nishimura et. al., 2004; Vivovac et. al., 1995). Isolated putative EVT progenitors were often present on the Matrigel surface in groups from day 2-3 of culture. Therefore, in order to determine whether these groups of cells were forming by proliferation of the putative EVT progenitors, or their migration towards each other, these cells were examined for the expression of Ki67. The majority of cells in these groups, which were morphologically distinct as trophoblasts, stained with Ki67 (Figure 4.10).

Figure 4.10 – Photomicrographs demonstrating the proliferation of putative EVT progenitors isolated from an 8 week placenta and immunostained after 4 days in culture. Groups of isolated putative EVT progenitors stained with the proliferation marker Ki67 (A&B). That these cells were trophoblasts was confirmed by immunostaining with cytokeratin (C) and vimentin (D). As Ki67 is a nuclear stain cells stained with this antibody were not counterstained with haematoxylin, however those stained with antibodies against cytokeratin (C) and vimentin (D) were counterstained with haematoxylin.
4.10 The expression of CD9 by putative EVT progenitors

Previous reports have suggested that fibroblasts can be immunodepleted from isolated trophoblasts using CD9 as a target (Morrish et. al., 1997; Verrijit et. al., 1997). However, some populations of isolated trophoblasts have been reported to express CD9 (Nagamatsu et. al., 2004b). Immunohistochemical analysis demonstrated that a significant proportion of putative EVT progenitors from 3 placentae were positive for CD9 after 3 days in culture (Figure 4.11). Fresh frozen sections of first trimester villous tissue were used to examine the expression of CD9 in vivo. These sections showed that mesenchymal cells in the core of large villi expressed CD9 (Figure 4.11, D) but CD9 was not expressed by all mesenchymal cells in smaller villi. Cytotrophoblasts in the monolayer underlying the syncytiotrophoblast did not express CD9, but groups of CD9 positive cells that may have been EVT progenitors were observed in some villous tips (Figure 4.11, C). Additional wells were stained with antibodies reactive with cytokeratin or vimentin to ensure cultures contained >90% trophoblasts.
Figure 4.11 – CD9 expression by putative EVT progenitors in situ. Putative EVT progenitors stained with antibodies reactive with A) CD9 or B) an irrelevant class matched control antibody. C) & D) Fresh frozen first trimester placental sections used as a positive control confirmed the expression of CD9 by some mesenchymal cells and groups of cells in villous tips that may have been EVT progenitors (arrow). Cells were counterstained with haematoxylin.

4.11 The differentiation of putative EVT progenitors in culture

In order to determine whether the putative EVT progenitors were differentiating into EVT or syncytiotrophoblast, putative EVT progenitors were cultured for 4 days and immunostained with antibodies reactive with markers of trophoblast differentiation throughout the four day culture period.
4.11.1 Syncytialisation of putative EVT progenitors

In order to determine whether the groups of putative EVT progenitors were syncytialising in culture, putative EVT progenitors cultured for 4 days were incubated with antibodies reactive with syncytiotrophoblast (G11) (Abumaree et. al., 2006). This showed that isolated trophoblasts were negative for G11 on day 4 of culture (Figure 4.12). The Jar choriocarcinoma cell line which has previously been shown to express the G11 antigen was used as a positive control (Figure 4.12, B) (Abumaree et. al., 2006). An irrelevant IgM class antibody (Jab-1) was used as a negative control and did not stain EVT outgrowths or isolated putative EVT progenitors (Figure 4.12, C). Additional wells were stained with antibodies reactive with cytokeratin or vimentin to ensure cultures contained >90% trophoblasts.

![Figure 4.12 – Phase contrast photomicrographs demonstrating staining of antibodies reactive with syncytiotrophoblast (G11) on A) putative EVT progenitors stained with the antibodies reactive with syncytiotrophoblast (G11). B) Positive controls of the Jar choriocarcinoma cell line. C) Negative controls of EVT outgrowth from a villous explant.](image-url)
4.11.2 Expression of HLA-G by putative EVT progenitors in situ

HLA-G is the only Class I MHC molecule expressed by EVTs and is absent from villous cytotrophoblasts. Replicate wells of isolated putative EVT progenitors were immunostained with an HLA-G reactive antibody after 24, 48, 72 or 96 hours of culture. HLA-G expression was not observed on day 1 or 2 of culture (Figure 4.13, A). However, from day 3 of culture onwards approximately 20% of trophoblasts expressed HLA-G (Figure 4.20). These HLA-G positive cells were often seen grouped with HLA-G negative trophoblasts (Figure 4.13, B).
Figure 4.13 – Photomicrographs showing HLA-G expression by isolated putative EVT progenitors (A,B). Putative EVT progenitors were also immunostained with vimentin (C) and cytokeratin (D). Fresh frozen first trimester placental sections used as a positive control confirmed the expression of HLA-G in EVTs only (E). Cells were counterstained with haematoxylin.
4.11.3 Expression of Class I MHC by isolated putative EVT progenitors in situ

In order to confirm the expression of HLA-G, separate, replicate wells of putative EVT progenitors isolated from 3 placentae were immunostained with a class I MHC reactive antibody (W6/32) after 24, 48, 72 or 96 hours of culture. Expression of Class I MHC was observed in the same pattern as that seen for HLA-G. No expression was seen on days 1 or 2 of culture. However, from day 3 of culture a few cells showed weak W6/32 reactivity (Figure 4.14, A). First trimester villous tissue stained with the W6/32 antibody at the same time confirmed Class I MHC expression was only observed in EVTs which had begun to migrate away from the villi (Figure 4.14, B).

Figure 4.14 – Photomicrographs demonstrating A) expression of class I MHC by putative EVT progenitors in situ. B) Class I MHC expression on sectioned first trimester villous tissue. C) The purity of putative EVT progenitors was determined with antibodies reactive with cytokeratin. D) Negative control of putative EVT progenitors stained with an irrelevant class matched antibody.
4.11.4 Putative EVT progenitor expression of the EVT marker Bo1D11

In order to further confirm that isolated trophoblasts were differentiating into EVTs, replicate wells of putative EVT progenitors isolated from 3 placentae were immunostained with antibodies reactive with EVTs (Bo1D11) after 96 hours in culture (Abumaree et al., 2006). At this time the majority of trophoblasts were Bo1D11 negative. However, a small proportion of cells, which were often located directly adjacent to the Bo1D11 negative cells, showed clear expression of Bo1D11, demonstrating that a limited number of cytotrophoblasts were differentiating into EVTs in these cultures (Figure 4.15 A, B). The Jar choriocarcinoma cell line which has previously been shown to express the Bo1D11 antigen (Abumaree et al., 2006), and EVT outgrowths from villous explants were used as positive controls. Putative EVT progenitors stained with the class matched antibody Jab-1 were used as negative controls (Figure 4.15, D). Additional wells of putative EVT progenitors were stained with antibodies reactive with cytokeratin or vimentin to ensure cultures contained >90% trophoblasts.
4.12 The effect of exogenous FGF-4 on EVT differentiation

In order to investigate previous reports claiming that exogenous FGF-4 was able to switch the cytotrophoblast differentiation pathway of bipotential cytotrophoblasts to favour the EVT lineage (Baczyk et. al., 2005), the effect of FGF-4 on outgrowth from villous explants, and on the differentiation of putative EVT progenitors was investigated.
4.12.1 The effect of FGF-4 on EVT outgrowth frequency

In order to determine the effect of exogenous FGF-4 on the production of EVT outgrowth 680 randomly selected explants from 6 placentae ranging from 6 to 10 weeks of gestation were cultured in either Complete Trophoblast Medium, or Complete Trophoblast Medium with 25ng/mL FGF-4 and 1µg/mL Heparin. Explants were cultured in 1.5% oxygen for 96 hours. The proportion of explants that produced EVT outgrowth (termed the frequency) was compared between cultures with (23.5%, n=80/340, p=0.52) and without (20.0%, n=68/340, P=0.52) exogenous FGF-4 and Heparin and was not significantly different (Figure 4.16).

EVT outgrowths in both cultures were only observed from villous tips, and no EVT outgrowths were evident from other regions of the villi that were in close proximity to the Matrigel surface (Figure 4.17).

![Histogram](Figure 4.16 – Histogram demonstrating the lack of significant difference (p=0.52) between the frequency of EVT outgrowth from villous explants cultured with Complete Trophoblast Medium or villous explants cultured with Complete Trophoblast Medium supplemented with 25ng/mL FGF-4 and 1µg/mL Heparin.)
4.12.2 The effect of FGF-4 on cell viability in villous explants

In order to determine whether the addition of exogenous FGF-4 and Heparin affected the viability or structure of villous explants, 42 explants from 6 placentae ranging from 7 to 10 weeks of gestation were stained with CMFDA and EtBr following 96 hours of culture in 1.5% oxygen in either Complete Trophoblast Medium (20 explants) or Complete Trophoblast Medium supplemented with FGF-4 and Heparin (22 explants). Both explants that had (19 explants), and explants that had not (23 explants) produced EVT outgrowth were examined. To ensure that the 1.5% oxygen was not affecting cell viability, control explants were incubated at 20% oxygen (Figure 4.18, C). No apparent differences in explant viability or structure were observed between explants in any of the treatment groups (Figure 4.18). The syncytiotrophoblast of all explants was non-viable as shown by its failure to metabolise CMFDA and its uptake of EtBr (Figure 4.18).
Some variation in the viability of the cytotrophoblast and mesenchymal cells was observed between villi within each explant, but the majority of cytotrophoblast and mesenchymal cells in most villi were viable.

Figure 4.18– Optical sections observed by confocal microscopy of first trimester villous explants stained with CMFDA and EtBr after 96 hours cultured in either 1.5% (A,B) or 20% oxygen in either Complete Trophoblast Media only (A,C) or Complete Trophoblast Media supplemented with FGF-4 and Heparin (B).
4.12.3 Putative EVT progenitor expression of FGFR-2

Whether isolated putative EVT progenitors expressed the FGF-4 receptor, FGFR-2, was assessed by immunohistochemical examination of isolated putative EVT progenitors from 3 placentae of 8 weeks gestation. This showed that after 96 hours of culture the vast majority of cells with trophoblast morphology expressed FGFR-2 (Figure 4.19). A blocking peptide was able to abolish the reactivity of the FGFR-2 antibody with the cells (Figure 4.19). Additional wells were stained with antibodies reactive with cytokeratin or vimentin to ensure cultures contained >90% trophoblasts.

![Photomicrographs demonstrating FGFR-2 expression by putative EVT progenitors](image)

Figure 4.19 – Photomicrographs demonstrating FGFR-2 expression by putative EVT progenitors A) Putative EVT progenitors stained with FGFR-2 antibodies. B) Putative EVT progenitors stained with the FGFR-2 antibody that had been preadsorbed with a blocking peptide C) Negative control of putative EVT progenitors stained with an irrelevant class matched antibody D) FGFR-2 expression in first trimester villous tissue.
4.12.4 The effect of FGF-4 on the differentiation of putative EVT progenitors

In order to determine whether FGF-4 acted on putative EVT progenitors to induce differentiation into EVTs, isolated putative EVT progenitors were cultured in either Conditioned Complete Trophoblast Medium (12 wells) or Conditioned Complete Trophoblast Medium supplemented with FGF-4 and Heparin (12 wells). On day 4 of culture cells were stained with antibodies reactive with HLA-G, and the percentage of HLA-G positive cells was determined in 5 random fields at 10x magnification in each well. There was no significant difference in the proportion of HLA-G positive cells between the treatment groups (p=0.74) (Figure 4.20).

Figure 4.20 – Histogram demonstrating that FGF-4 had no significant effect on putative EVT progenitor differentiation into EVT.
4.13 Summary of Key Findings

Work in this chapter has described the identification, isolation and characterisation of a subpopulation of cytotrophoblasts that appear to be committed to EVT differentiation. The key findings were:

- Despite the death of the vast majority of cytotrophoblasts by 48 hours in explant culture, cytotrophoblasts located in the cell islands in villous tips were able to remain viable for at least 2 weeks in culture, and produce new EVT outgrowth.
- A population of putative EVT progenitors were isolated from first trimester villous explants after 10 days in culture.
- Putative EVT progenitors expressed $\alpha_\nu\beta_6$ integrin, which is a marker of cytotrophoblasts in the cell islands of anchoring villi \textit{in vivo}.
- Putative EVT progenitors did not produce tenascin.
- Putative EVT progenitors proliferated \textit{in vitro}.
- Putative EVT progenitors did not form syncytiotrophoblast within 96 hours of culture.
- Approximately 20\% of putative EVT progenitors differentiated into EVTs within 96 hours of culture, but the remainder appeared to stay undifferentiated.
- Addition of exogenous FGF-4 to villous explant cultures did not significantly alter the frequency of EVT outgrowth produced, or the viability of the explants.
- Putative EVT progenitors expressed FGFR-2.
- Addition of exogenous FGF-4 did not affect the proportion of putative EVT progenitors that differentiate down the EVT lineage as shown by the expression of HLA-G.
4.14 Discussion

4.14.1 Overview

In Chapter 3 it was demonstrated that many, if not most, cells in placental villi die relatively early during explant culture. However, the evidence in this chapter shows that new EVT outgrowths were able to be produced for up to three weeks of culture due to the prolonged survival of cytotrophoblasts present in multilayered clusters in the tips of villi from which EVT outgrowth is produced. The extended viability of this group of cells in explant culture was exploited to develop a novel method of isolating this specific population of putative EVT progenitors, and these cells were characterized in vitro.

4.14.2 Cytotrophoblasts in the first trimester are not a single bipotent population.

Since most cells in explanted villi, including the monolayer of cytotrophoblasts underlying the syncytiotrophoblast, had died within a week of culture, it was somewhat surprising that anchoring villi could continue to produce EVT outgrowths for up to three weeks. However, this work has demonstrated the production of EVT outgrowth from villi in which the vast majority of the villous was non-viable. This meant that the expansion of EVT columns from these villi could not be dependent on either the cells of the mesenchymal core or the majority of the villous cytotrophoblasts, which are largely non-viable after the first week of culture. In contrast to floating villi, anchoring villi contain multiple layers of villous cytotrophoblasts at their tips, which are termed cell islands (Vivovac et al., 1995). It has been shown in this work that these multilayered cell islands at the origins of trophoblast columns remain viable during prolonged culture. It is widely accepted that villous cytotrophoblasts from term placentae are committed to differentiate into syncytiotrophoblast (Kliman et al., 1986; Morrish et al., 1997), but that early gestation villous cytotrophoblasts are bipotential. Based on the evidence in this thesis, it is proposed that, as early as 8 weeks of gestation, there are two distinct populations of villous cytotrophoblasts:

1) The majority of villous cytotrophoblasts form a monolayer directly beneath the syncytiotrophoblast. These cells do not survive well in the culture conditions employed in this work and it is likely that they are committed to fusion into
syncytiotrophoblast. These cells will be referred to as monolayer villous cytotrophoblasts.

2) Villous cytotrophoblasts that are committed to the EVT differentiation pathway are located in multilayered pockets at anchoring villous tips. This type of villous cytotrophoblast will be referred to as EVT progenitors.

The lines of evidence for the existence of a separate sub-population of EVT progenitors are firstly, although the vast majority of villous cytotrophoblasts that underlie the syncytiotrophoblast were non-viable within the first week of culture, EVT outgrowth was produced from explants for up to three weeks in culture. Although it is a possibility that the increased survival capacity of EVT progenitors was a result of differences in the local environment of these cells, it seems unlikely that this explanation could account for prolonged survival of EVT progenitors for more than a few days. Whereas, it has been shown here that EVT progenitors survived (as demonstrated by the production of new EVT outgrowth, and their metabolism of CMFDA and exclusion of EtBr) for more than two weeks after the death of the cytotrophoblasts in the monolayer. It should be noted that, while new EVT outgrowths were generated, there was no regeneration of the syncytiotrophoblast in the region around the anchoring tips (Figures 4.1 and 4.4) suggesting that the cytotrophoblasts in anchoring tips do not differentiate into syncytiotrophoblast, and are not bipotent. Therefore, these cells may be fundamentally different to monolayer villous cytotrophoblasts with an increased ability to survive in culture, allowing the formation of EVT outgrowths in explants when the villous monolayer cytotrophoblasts are no longer viable. The EVT progenitors were more resilient in culture, which may reflect an important physiological adaptation by the EVT progenitors to equip EVTs for the physical environment they are exposed to in vivo when migrating from the placenta.

Secondly, this work revealed that although all explants were in contact with a permissive ECM, a significant number of explants produced EVT outgrowths in a secondary passage culture but not in the primary culture. It has previously been reported that contact with a permissive ECM is sufficient to stimulate trophoblast outgrowth and differentiation from explanted first trimester villi in vitro (Aplin et. al., 1999). The results presented here suggest that the reorientation of the explants in the subsequent passage
cultures brought a villous tip containing viable EVT progenitors into contact with the Matrigel, resulting in a new site of outgrowth formation.

Thirdly, it has been suggested that growth factors from the villous mesenchymal core, villous cytotrophoblasts and syncytiotrophoblast play a significant role in the formation and expansion of EVT outgrowth (Bischof et al., 2000; Lacey et al., 2002). However, in this work despite the death of the vast majority of cells in the villi, EVT outgrowth continued for up to three weeks, albeit at a reduced rate. This suggests that EVT progenitors are not solely dependent upon paracrine signals to drive their differentiation or invasive capacity. However, it is likely that paracrine factors would enhance trophoblast outgrowth. Alternatively, sufficient growth factors may be derived from the Matrigel, which although used as a very thin coat of diluted (10%) Matrigel on the culture wells in this model, remains a potential source of cytokines and growth factors. The behaviour of cells in culture is dependent on the conditions of culture employed. Many workers use three-dimensional models with deep layers of concentrated Matrigel. In these other models it is possible that relatively large quantities of factors that promote cell survival or other cellular behaviour are supplied by the Matrigel and thus the results from the model used in this work may not be directly compatible with other models which use much larger amounts of Matrigel. Finally, the observed decrease in the frequency of EVT outgrowth with gestation demonstrated in Chapter 3 indicates that, in the first trimester, as gestation progresses, more floating villi are formed but these are intrinsically different to anchoring villi and they do not have the potential to produce EVT.

The lack of a suitable human cell culture models in which cytotrophoblasts differentiate down the EVT lineage has hampered the understanding of the factors that control EVT differentiation and outgrowth. The identification of a population of cytotrophoblasts committed to extravillous trophoblast differentiation would therefore be an appealing target by which to establish such a model. Therefore, the extended viability of this population was used in order to isolate these cells from first trimester placentae and study them in greater detail.
4.14.3 The isolation of putative EVT progenitors from explanted villi.

The differentiation of cytotrophoblasts into EVT is a crucial process for the success of implantation. Cytotrophoblasts isolated from fresh first trimester placentae have been used to study this process, but this culture model has been confounded by problems with syncytialization, aggregation or detachment of the cells from the culture plate (Aboagye-Mathiesen et. al., 1996; Nagamatsu et. al., 2004a). Furthermore, the morphology and phenotype of isolated cytotrophoblasts appears to be dependent on the gestation of the placenta, the length of treatment with trypsin, and the use of density gradient separation or immuno-absorptive depletion with antibodies against cell populations other than cytotrophoblasts such as anti-CD9, anti-laminin or anti-CD90 (Aboagye-Mathiesen et. al., 1996; Loke et. al., 1986; Loke et. al., 1989; Morrish et. al., 1991; Nagamatsu et. al., 2004a; Yui et. al., 1994). The hypothesis that there is a separate cytotrophoblast sub-population of EVT progenitors presented in the previous section also raises a further issue in the use of isolated cytotrophoblasts to study EVT differentiation – that the cytotrophoblasts obtained by conventional methods of cytotrophoblast isolation may infact not be the correct cell population to target in order to accurately study EVT differentiation in vitro. Indeed, the tendency of cytotrophoblasts isolated from first trimester placentae to syncytialise in culture (Morrish et. al., 1997) suggests as might be expected that the majority of those cells are infact monolayer villous cytotrophoblasts committed to differentiation into syncytiotrophoblast. Therefore, in order to obtain a population of relevant EVT progenitors for potential use as a model of EVT differentiation in vitro, the prolonged viability of EVT progenitors in comparison to the other cell types in the villous was used to develop a novel methodology of isolating these cells from first trimester villous explants. Sequential trypsinization of villous explants which had been in culture for 10 days produced a viable population of trophoblast cells. As these cells were shown to be the only cells viable in the villi at the time of trypsinization, it is likely that the viable trophoblast population obtained were those from the villous tips, and thus have been termed putative EVT progenitors.

Others have previously reported that CD9 can be used to immunodeplete fibroblasts from trophoblast isolates (Morrish et. al., 1997; Verrijit et. al., 1997). A low level (<10%) of fibroblast contamination was observed in putative EVT progenitor isolates obtained in this work. Despite preliminary attempts using magnetic beads to improve the purity of putative EVT progenitors, a CD9 immunodepletion step was not used to remove
fibroblasts in cultures of isolated putative EVT progenitors for two reasons. Firstly, the isolated putative EVT progenitors obtained had been shown to express CD9 \textit{in vitro}, and CD9 expression was observed in groups of cells in villous tips of fresh frozen first trimester sections, suggesting that CD9 expression may have been present in the EVT progenitors at the time of digestion. Thus, using CD9 as a target for depletion may have potentially selected against this target trophoblast population. Secondly, the use of immunomagnetic beads appeared to substantially reduce the number of trophoblasts obtained at the end of the protocol, possibly due to the additional steps in the procedure affecting the viability of the cells being isolated, or as a result of a previously reported effect of non-specific adhesion of trophoblast cells to the immunomagnetic beads that results in a loss of some trophoblasts (Aboagye-Mathiesen \textit{et. al.}, 1996). Despite this lack of specific fibroblast depletion, and even though the number of fibroblasts in villi are much greater than the number of EVT progenitors, the level of initial fibroblast contamination was extremely low, and the majority of cultures contained >95% pure trophoblasts 24 hours after isolation. The presence of these contaminating fibroblasts in the isolated putative EVT progenitors demonstrates that not all cells in the villous mesenchymal core died during prolonged explant culture. However, as discussed in Chapter 3 the analysis of cell death in explants was confined to the small terminal villi that are likely to produce extravillous trophoblast outgrowth. Technical limitations prevented the extension of that analysis to larger stem villi and it seems likely that the small number of contaminating fibroblasts came from such larger villi.

\textbf{4.14.4 Evidence that the isolated putative EVT progenitors are derived from the trophoblast cell islands in anchoring villi.}

The major lines of evidence in this thesis that indicated that putative EVT progenitors that have been isolated after extended explant culture are not bipotent, and are the same as the EVT progenitor population that I have identified \textit{in vivo} are that:

1) Multilayered groups of EVT progenitors in villous tips were the only viable cells in the villi after 10 days of explant culture, and these did not induce regeneration of the surrounding syncytiotrophoblast, but were able to form EVT outgrowths (Figure 4.1, Figure 4.2).

2) Isolated putative EVT progenitors stained strongly with antibodies reactive with \(\alpha v\beta 6\) integrin. \(\alpha v\beta 6\) integrin is only expressed by villous cytotrophoblasts at sites
of EVT column initiation, and not in monolayer cytotrophoblasts or other cell types in the villi (Zhou et al., 1997).

3) The multilayered cytotrophoblasts in the cell islands at the tips of anchoring villi proliferate in order to drive the expansion of the EVT columns, and a high proportion stain with the proliferation marker Ki67 in comparison to the majority of monolayer villous cytotrophoblasts underlying the syncytiotrophoblast (Korhonen & Virtanen, 1997; Vivovac et al., 1995). After several days of culture putative EVT progenitors were present in small groups of cells which were actively proliferating, as shown by their expression of Ki67. This highlights the difference between these putative EVT progenitors, and those isolated from fresh first trimester placentae which are predominantly monolayer villous cytotrophoblasts and do not proliferate in culture, but rather differentiate into syncytium (Morrish et al., 1997).

4) Putative EVT progenitors did not syncytialize during 4 days of culture in vitro. Cytotrophoblasts isolated from fresh first trimester placentae undergo syncytialization within 1-2 days of culture (Morrish et al., 1997; Tarrade et al., 2001). In contrast, isolated EVT progenitors failed to stain with the syncytial marker G11, showing that they did not syncytialise over 4 days in culture. Syncytialised trophoblasts do not actively proliferate (Boyd & Hamilton, 1970). Therefore, the expression of Ki67 in these groups of cells further confirms that the putative EVT progenitors did not syncytialize.

5) A proportion of isolated putative EVT progenitors differentiated into EVTs. By the end of the 4 day culture period examined approximately 20% of cells stained with antibodies reactive with the EVT markers HLA-G, Class I MHC and Bo1D11. These cells were sometimes observed adjacent to non-staining cells on the plate. This suggests that a proportion of putative EVT progenitors were differentiating into EVTs. Furthermore, the existence of adjacent, non-staining cells suggests that many isolated EVT progenitors remain as undifferentiated progenitor cells in culture.

Tenascin, an ECM glycoprotein, is present in anchoring villi immediately adjacent to sites of cytotrophoblast column initiation, but is not detected on monolayer villous cytotrophoblasts (Castellucci et al., 1991; Damsky et al., 1992). However, while putative EVT progenitors expressed the receptor for tenascin, αvβ6 integrin, they did not stain
with antibodies reactive with the ECM protein tenascin. It is not clear whether the tenascin expression which has been associated with EVT progenitors in vivo is produced by these cells or by adjacent fibroblasts, possibly as a result of paracrine interactions with the EVT progenitors. Therefore, the lack of tenascin expression by putative EVT progenitors in vitro does not detract from the evidence that the putative EVT progenitors that have been isolated are likely to be the population identified as EVT progenitors in villous explants.

4.14.5 Previous evidence that there are two separate populations of cytotrophoblasts in first trimester placentae

My hypothesis that a distinct population of EVT progenitors exists has support from the literature. Isolated cytotrophoblast populations from first trimester placentae that have the ability to differentiate down the EVT pathway have been previously reported (Aboagye-Mathiesen et. al., 1996; Nagamatsu et. al., 2004a; Tarrade et. al., 2001). Aboagye-Mathiesen et. al. (1996) describe the isolation of four distinct trophoblast populations that they obtained by collecting different cell fractions from sequential 10 minute trypsinization of fresh first trimester placentae (Aboagye-Mathiesen et. al., 1996). Cells collected after 10 minutes of trypsinization were described as mononuclear villous cytotrophoblasts that become committed to syncytium formation. In contrast, cells collected after 30 minutes of trypsinization consisted of a population of trophoblasts which appear in a ‘crazy pavement’ pattern and expressed markers of EVTs including $\alpha 1$ integrin, E-cadherin and Class I MHC in vitro (Aboagye-Mathiesen et. al., 1996).

Furthermore, a significant difference in cytotrophoblasts obtained from placentae of 10 weeks of gestation or less, or placentae greater than 10 weeks of gestation was observed (Aboagye-Mathiesen et. al., 1996). In placenta of 10 weeks of gestation or less, in addition to the population of ‘crazy pavement’ trophoblasts obtained after 30 minutes of trypsinization, on a few occasions trophoblasts with a flattened appearance that proliferated very slowly in culture were also obtained (Aboagye-Mathiesen et. al., 1996). In contrast, in placentae greater than 10 weeks of gestation, even after 30 minutes of trypsinization, the majority of cells obtained were villous trophoblasts which had no ability to proliferate, and differentiated to form multinucleated syncytiotrophoblast in vitro (Aboagye-Mathiesen et. al., 1996). The cells obtained from placentae of 10 weeks of gestation and less are described by Aboagye-Mathiesen et. al. as EVTs based on their
molecular phenotype, but it seems unlikely that EVTs already present in outgrowths from the placental tissue would remain after this length of trypsinization, particularly when villous cytotrophoblasts were obtained after only 10 minutes of trypsinization. Therefore, in light of the identification of a population of EVT progenitors in this work, it may be possible that the cells described by Aboagye-Mathiesen et al. were derived either from trophoblasts that had already begun EVT differentiation in the villous tips, or by EVT progenitors that rapidly differentiated in the culture conditions they employed. Of particular interest were the slowly proliferating trophoblasts obtained from some placentae, which may potentially have been similar to the isolated putative EVT progenitor population described in this work. The difference in the cytotrophoblast populations obtained at different gestational ages is in line with the results in chapter 3 of this thesis demonstrating that the proportion of cytotrophoblasts with the potential to form EVT outgrowth decline with increasing gestation.

The methodology used in this work differs from that developed by Aboagye-Mathiesen et al. in several ways. Firstly, in this work, the starting material was villi from placentae of less than 10 weeks of gestation that had been maintained in explant culture for 10 days, whereas Aboagye-Mathiesen et al. used fresh placentae (Aboagye-Mathiesen et al., 1996). Secondly, cells obtained from sequential trypsinizations in this work were pooled. Some preliminary experiments were performed in which cells from sequential trypsinic digests of cultured explants were cultured separately, however very few cells were obtained after 10 minutes of trypsinization, and no difference in the cell populations obtained from the 2nd to the 5th sequential 10 minute digests was discernable. Therefore, the methodology that produced the best yield of cells, i.e. discarding the first 10 minute digest and pooling the remaining digs, was adopted. The absence of viable cells in the 10 minute trypsinic digest in which Aboagye-Mathiesen et al. obtained primarily villous cytotrophoblasts that were committed to syncytialization confirms that the vast majority of monolayer villous cytotrophoblasts were non-viable at the time of trypsinization (Aboagye-Mathiesen et al., 1996). Thirdly, as the target population of EVT progenitors had been shown to be the only viable cells in the villi at the time of trypsinization, unlike Aboagye-Mathiesen et al., a percol gradient was not used to separate out these cells, but rather the non-viable cells were washed from the Matrigel surface after 24 hours (Aboagye-Mathiesen et al., 1996).
Using an adaptation of the method of Aboagye-Matheisen et al. with an altered density combination for the percoll gradient, Nagamatsu et al. (2004) have also isolated a population of cytotrophoblasts, from placentae of 10 weeks of gestation or less, that have similar properties to those in trophoblast cell islands in anchoring villous tips (Nagamatsu et al., 2004a). In vitro, the isolated trophoblasts obtained by Nagamatsu et al., gradually upregulated α1 integrin, HLA-G and CD9, and did not syncytialise (Nagamatsu et al., 2004a). Likewise, the putative EVT progenitors isolated in this work did not syncytialise, expressed CD9 after 3 days in culture, and a proportion of the cells also upregulated HLA-G after 2-3 days in culture.

Taken together, the results I have presented in this work plus the results of Aboyage-Mathisen et al. strongly suggest that even in the first trimester not all villous cytotrophoblasts are identical and there appears to be at least two separate populations of villous cytotrophoblasts, one committed to syncytialization, and the second committed to the EVT pathway.

### 4.14.6 Evidence from the literature for a bipotential villous cytotrophoblast population

Despite the widely held belief that cytotrophoblasts are bipotential in the first trimester, there is little direct evidence in the literature demonstrating this bipotential behaviour. However, one recent publication claimed to show “true bipotential behaviour of a subset of cytotrophoblasts residing on the basal lamina of villi” (Baczyk et al., 2005). Baczyk et al. described experiments in which they removed the syncytiotrophoblast from placentae of 11-12 weeks gestation. In control conditions, the cytotrophoblasts of denuded villi differentiated to reform syncytiotrophoblast. In contrast, denuded villi cultured with FGF-4 failed to regenerate the syncytiotrophoblast (Baczyk et al., 2005). In these FGF-4 treated villi, proliferation of FGFR-2 positive cytotrophoblasts was stimulated, purportedly resulting in the formation of ‘cytotrophoblast clumps’, which exhibited invasive markers indicative of differentiation into EVT (Baczyk et al., 2005). From these results Baczyk et al. concluded that FGF-4 redirected differentiation of cytotrophoblasts to an EVT phenotype rather than to syncytialization, and therefore that a population of bipotential cytotrophoblasts exists (Baczyk et al., 2005). While Baczyk et al. did demonstrate that FGF-4 inhibits syncytialization of villous cytotrophoblasts, their data
does not demonstrate that these same cytotrophoblasts were bipotent and induced by FGF-4 to adopt an extravillous phenotype. Baczyk et al. examined only placentae of 11-12 weeks gestation. Such placentae contain many anchoring villi from which EVT columns arise in vivo. In such villi the trophoblast columns form adjacent to multilayered cell islands of proliferating villous cytotrophoblasts that have a similar appearance to the “cytotrophoblast clumps” supposedly induced by FGF-4 in the study of Baczyk et al. It is unclear how Baczyk et al. were able to distinguish between pre-existing anchoring villous tips and the “cytotrophoblast clumps” which they claim were formed de novo in denuded villi as a consequence of FGF-4 treatment. Furthermore, if the villous cytotrophoblasts which underlie the denuded syncytiotrophoblast were bipotent then it would be expected that “cytotrophoblast clumps” would form around large areas of the denuded villi at all points were the cultured villi contacted the growth surface as suggested by Aplin et al. (Aplin et al., 1999). Finally, it is interesting that although Baczyk et al. report an induction of differentiation down the EVT lineage by FGF-4, this contrasts directly with studies on the effect of FGF-4 on trophoblast stem cells derived from mice, which have shown that FGF-4 inhibits trophoblast stem cell differentiation, and was infact added to culture in order to maintain the trophoblast stem cell phenotype (Guzman-Ayala et al., 2004; Hughes et al., 2004; Lei et al., 2007; Tanaka et al., 1998). In contrast to the findings of Baczyk et al., the data presented here shows that addition of exogenous FGF-4 to villous explants of less than 10 weeks gestation did not result in a significantly increased frequency of EVT outgrowth, or in the formation of outgrowths from areas of the villous other than the villous tips, in which EVT progenitor population identified in this chapter would be expected to be located. Furthermore, although putative EVT progenitors expressed FGFR-2, the addition of exogenous FGF-4 did not discernibly alter the number of these cells in culture, or the proportion of cells differentiating down the EVT lineage.

The data presented in this thesis refute Baczyk et al. ‘s interpretation of their results that a single population of bipotential villous cytotrophoblasts exists. Therefore, I propose an alternative model of the actions of FGF-4 on cytotrophoblast differentiation that may also be inferred from the data of Baczyk et al. This model is that FGF-4 may have different actions on two distinct villous trophoblast subpopulations in vivo – acting to stimulate proliferation of FGFR-2 positive EVT progenitors, while inhibiting the fusion of monolayer villous cytotrophoblasts into syncytiotrophoblast.
4.14.8 The consequences for placental research of the existence of ‘EVT progenitors’

The finding that villous cytotrophoblasts from first trimester placentae are not bipotent progenitors has several implications for the field of placental research. Firstly, this may explain why it is difficult to obtain large numbers of trophoblasts that either differentiate into an invasive EVT phenotype or proliferate following enzymatic digestion of first trimester placentae, as the vast majority of villous cytotrophoblasts are contained in the villous monolayer and would be committed to the syncytiotrophoblast differentiation pathway. Therefore, results using cytotrophoblasts isolated from fresh first trimester placentae using the traditional ‘Kliman’ (Kliman et. al., 1986) enzymatic digestion methods, should be interpreted with this in mind.

Secondly, the isolation and culture of putative EVT progenitors may provide a more accurate in vitro model for the study of trophoblast differentiation along the EVT lineage. It would have been advantageous to exploit the proliferation observed over the first few days of culture to create an ongoing cell line in this work. However, the continued proliferation of these cells appeared to be affected by the culture conditions. Despite the initial burst of proliferation in culture, the putative EVT progenitors did not respond well to passaging, and did not proliferate when plated at low cell densities. The self-renewal rate of stem cell populations in culture is typically low, and population doubling times in isolated stem cell populations can increase significantly as cultures progress (Izadpanah et. al., 2006; Lee et. al., 2006). In addition, mouse trophoblast stem cells have been shown to be very sensitive to the culture conditions employed and this has the potential to affect their survival, differentiation and proliferation (Lei et. al., 2007). Therefore, due to these factors, and the fact that cultures contained very low levels of rapidly proliferating fibroblasts which result in an unacceptable level of contamination with extended culture, it was not possible within the time frame of this work to obtain a pure population of putative EVT progenitors.

4.14.9 Summary

Despite the extensive cell death of the majority of villous cytotrophoblasts underlying the cytotrophoblast and cells of the mesenchymal core during the first week of culture, multilayered EVT progenitors in the villous tips remained viable, and explants retained
the capacity to produce new EVT outgrowth for up to three weeks. Therefore the hypothesis has been formed that first trimester villi contain two distinct villous cytotrophoblast populations that are committed to differentiate either into syncytiotrophoblast or EVTs. The prolonged survival of EVT progenitors in villous tips was exploited in order to isolate a putative population of these cells from explanted villi. This resulted in the isolation of a viable population of trophoblasts which exhibited similar characteristics to those found in cell islands in villous tips in vivo. In addition, these cells did not syncytialize, and a proportion differentiated into EVT over 4 days in culture. Neither EVT outgrowth from villous explants, nor differentiation of isolated putative EVT progenitors, were affected by the addition of FGF-4, despite the isolated putative EVT progenitors expressing FGFR-2. This suggests that FGF-4 is not able to direct cytotrophoblast differentiation into EVT in this model, and provides further evidence to suggest that cytotrophoblasts in the first trimester are not a homogenous bipotent population. From the results in this chapter I conclude that it is likely that the isolated putative EVT progenitor population are indeed EVT progenitors.
Chapter Five:

The Effect of Oxygen on Extravillous Trophoblast Outgrowth
5.1 Introduction and rationale

It is now apparent that during the first 10 weeks of pregnancy, EVTs form plugs in the uterine spiral arteries, occluding the flow of maternal blood to the IVS (Hustin & Schaaps, 1987; Irving & Lala, 1995a; Jaffe & Woods, 1993; Jauniaux et. al., 2003b; Jauniaux et. al., 1992). Thus, for the majority of the first trimester, the placenta develops in conditions of physiological hypoxia in which the local oxygen concentration is as low as 1-2% (Rodesch et. al., 1992). The trophoblast plugs are believed to progressively disperse from 10 to 12 weeks of gestation, allowing maternal blood to flow into the IVS (Hustin & Schaaps, 1987; Jaffe & Woods, 1993; Jauniaux et. al., 2003b; Jauniaux et. al., 1992). Contemporary theory holds that the low oxygen conditions during the first 10-12 weeks of pregnancy regulate placental development and EVT outgrowth. Therefore, in order to determine the effect of low oxygen concentrations on the frequency, area and phenotype of EVT outgrowths, trophoblast outgrowth from first trimester villous explants cultured in either 1.5% or 8% oxygen was characterised in the following work.

5.2 The effect of oxygen concentration on the frequency of extravillous trophoblast outgrowth from cultured explants

In this work 3963 randomly selected explants from 25 placentae evenly distributed from 8 to 12 weeks of gestation were cultured in either 1.5% or 8% oxygen. The large number of explants allowed the entire placenta, containing both anchoring and floating villi, to be represented. Therefore only a certain percentage of villi in any culture produced EVT outgrowth, and this allowed the number of villi producing EVT outgrowth to be quantified (outgrowth frequency). In order to determine the effect of oxygen concentration on the number of explants producing EVT outgrowths, the frequency of EVT outgrowth was compared between explants cultured in 1.5% oxygen or 8% oxygen (see Appendix B for details on the low oxygen culture system used). Significantly fewer (p<0.001) explants produced EVT outgrowths in 1.5% oxygen (14%, n = 239/1713) than in 8% oxygen (18.6%, n = 317/1702) (Figure 5.1).
5.3 The effect of gestational age on the differential response of trophoblast outgrowth to oxygen

In order to examine whether the effect of oxygen concentration on EVT frequency applied equally to placentae of different gestations, the differential response of villous explants to oxygen concentration was examined by determining the difference in the percentage of explants that produced outgrowths in matched cultures of explants from the same placenta grown in 1.5% or 8% oxygen. The term “differential response” was coined to describe this and is calculated using the formula: differential response = [frequency of EVT outgrowth in 8% oxygen] – [frequency of EVT outgrowth in 1.5% oxygen].

There was a significant difference (p=0.0005) between the differential response of explants from the same placentae to oxygen concentration for placentae under 11 weeks of gestation (8.4%, n=15 placentae), and the differential response observed from explants from placentae of 11 weeks gestation and greater (0.79%, n=10 placentae) (Figure 5.2). The alteration in differential response to oxygen concentration with
gestational age was not a gradual trend but rather there was a discrete change that occurred between 10 and 11 weeks of gestation (Figure 5.2).

Figure 5.2 - Histogram demonstrating the difference in the percentage of EVT outgrowths formed between 8% and 1.5% oxygen conditions. Explants from 5 placentae at each gestational age were cultured in 8% oxygen, and similar numbers of explants from the same placentae were cultured in 1.5% oxygen for 5 days. The percentage of explants producing EVT outgrowth was determined, and the differential response of each placenta to oxygen was calculated. The change in the data by gestational week is significant by ANOVA (p=0.012).

5.4 The effect of oxygen concentration on the area of extravillous trophoblast outgrowth from villous explants

In order to determine the effect of oxygen concentration on the size of EVT outgrowths, the area of outgrowths from explants cultured in either 1.5% or 8% oxygen was analyzed. The mean area of EVT outgrowths produced in 1.5% oxygen (0.94mm², n=238), was significantly less (p=0.028) than in 8% oxygen (1.26mm², n=317) (Figure 5.3). There was no significant difference in outgrowth area with gestational age. In order to determine whether the decrease in EVT outgrowth area in 1.5% oxygen was a result of declining cell viability, outgrowths were stained with the viability marker trypan blue. EVTs in outgrowths produced in both 1.5% oxygen and 8% oxygen were viable by trypan blue exclusion (Figure 5.4).
Figure 5.3 – Histogram demonstrating the difference in EVT outgrowth area from first trimester villous explants cultured in 1.5% or 8% oxygen. *p=0.028.

Figure 5.4 – Photomicrographs of EVT outgrowths from an 8.5 week placenta cultured for 4 days in either 8% (A) or 1.5% (B) oxygen and stained with trypan blue. The villous explant can be seen in the lower left corner of each image.
5.5 The effect of oxygen concentration on the size of individual extravillous trophoblasts

In order to determine whether the increase in EVT outgrowth area was due to an increase in trophoblast size, the area of 400 individual EVTs in outgrowths from explants cultured in 1.5%, and 400 individual EVTs from explants cultured in 8% oxygen, was determined using Image J. The mean area of EVTs cultured in 1.5% oxygen (n=400) was 23.7% smaller (p<0.001) than those in 8% oxygen (n=400). Further investigation revealed that regardless of oxygen concentration, EVTs in the regions of the cell columns distal to the villi (n=400) were 41.2% larger (p<0.001) than those in the regions proximal to the villi (n=400) (Figure 5.5). The increase in cell area between 1.5% oxygen and 8% oxygen conditions was evident in both the proximal and distal regions of the cell columns. EVTs in the proximal regions of the outgrowths cultured in 1.5% oxygen had an area 28.1% smaller (p<0.001) than those in the proximal regions of outgrowths cultured in 8% oxygen. Whereas, EVTs in the distal regions of the outgrowths cultured in 1.5% oxygen had an area 20.1% smaller (p<0.001) than those in the distal regions of outgrowths cultured in 8% oxygen (Figure 5.5).

![Figure 5.5 – Histogram demonstrating the difference in EVT area in proximal (blue bars) and distal (green bars) regions of EVT outgrowth under 1.5% and 8% oxygen conditions. * p<0.001; ** p<0.001.](image)
5.6 The effect of oxygen concentration on the expression of HLA-G by extravillous trophoblasts in outgrowths from villous explants

In order to determine if oxygen concentration altered the phenotype of EVTs, outgrowths from explants cultured in 1.5% or 8% oxygen were stained \textit{in situ} with antibodies reactive with HLA-G. HLA-G was expressed by EVTs throughout the outgrowths in both 1.5\% (n = 12) and 8\% (n = 10) oxygen cultures and there were no apparent differences in the level of expression of HLA-G by EVTs grown in 1.5\% or 8\% oxygen (p = 0.38) (Figure 5.6).

Figure 5.6 – Photomicrographs of EVT outgrowths from villous explants cultured in 1.5\% (A) and 8\% (B) oxygen and stained \textit{in situ} with an antibody reactive with HLA-G. Staining was performed after 5 days of culture. No staining was observed with isotype matched antibodies reactive with vimentin (C). Counterstained with haematoxylin.
5.7 The effect of oxygen on the expression of α₁ integrin in extravillous trophoblasts in outgrowths from villous explants

In order to further examine the effect of oxygen concentration on EVT phenotype, outgrowths from explants cultured in 1.5% or 8% oxygen were stained with antibodies reactive with α₁ integrin, an adhesion molecule involved in EVT invasion (Damsky et. al., 1994). α₁ integrin was expressed by EVTs throughout the outgrowths in both 1.5% (n = 12) and 8% (n = 14) oxygen and there were no apparent differences in the level of expression of α₁ integrin by EVTs grown in 1.5% or 8% oxygen (p = 0.44) (Figure 5.7).

Figure 5.7 – Photomicrographs of EVT outgrowths from villous explants cultured in 1.5% (A) and 8% (B) oxygen and stained in situ with an antibody reactive with α₁ integrin. Staining was performed after 5 days of culture. No staining was observed with isotype matched antibodies reactive with vimentin (C). Counterstained with haematoxylin.
5.8 The effect of oxygen concentration on the expression of p27 in extravillous trophoblasts in outgrowths from villous explants

In order to determine if oxygen concentration altered the cell cycle progression of EVTs as they differentiate into an invasive trophoblast phenotype, outgrowths from villous explants cultured in 1.5% or 8% oxygen were stained with antibodies reactive with p27, a cell cycle inhibitor reported to be upregulated as trophoblasts differentiate into an invasive phenotype (Korgun et al., 2006). Nuclear p27 expression was inconsistent and no significant difference in the number of EVT outgrowths showing p27 expression was observed between explants cultured in 1.5% oxygen (5/11 outgrowths) and 8% oxygen (3/11 outgrowths, p = 0.09). There were no apparent differences in the pattern of p27 expression by EVTs grown in 1.5% or 8% oxygen (Figure 5.8). Expression of p27 was only observed in the nuclei of some EVTs, predominantly in those cells at the edges of the outgrowths (Figure 5.8).

Figure 5.8 – Photomicrographs of EVT outgrowths from villous explants stained with an antibody reactive with p27. Staining was performed in situ after 5 days of culture in either 1.5% (A) or 8% (B) oxygen. Staining was observed with isotype matched antibodies reactive with cytokeratin (C), but not with isotype matched antibodies reactive with vimentin (D).
5.9 Summary of key experimental findings

Work in this chapter has analysed EVT outgrowths produced by first trimester villous explants in response to culture in 1.5% or 8% oxygen. The key findings were:

- Villous explants cultured in 1.5% oxygen produced significantly fewer EVT outgrowths than those cultured in 8% oxygen.

- The differential response in the production of EVT outgrowth between 1.5% oxygen and 8% oxygen was dependent on the gestational age of the tissue, with villous explants from placentae of 8-10 weeks of gestation having a significant differential response to oxygen, whilst villous explants from placentae of 11-12 weeks of gestation did not have a significant differential response to oxygen, i.e. oxygen concentration did not affect the frequency of trophoblast outgrowth from placentae of 11-12 weeks gestation.

- EVT outgrowth area in this model was able to be accurately quantified.

- EVT outgrowths from villous explants cultured in 1.5% oxygen had a significantly smaller total area than those produced in 8% oxygen.

- Individual EVTs in outgrowths formed in 1.5% oxygen were significantly smaller than those in outgrowths formed in 8% oxygen.

- Individual EVTs in regions of EVT outgrowths proximal to the villi had a smaller area than those in regions of EVT outgrowths distal to the villi.

- Oxygen concentration did not affect the expression of HLA-G by EVTs.

- Oxygen concentration did not affect the expression of α1 integrin by EVTs.

- Less than 50% of outgrowths contained trophoblasts that exhibited nuclear expression of p27, and expression was only observed in those EVTs in the distal region of the outgrowth. No difference in the staining pattern for p27 was observed between 1.5% and 8% oxygen conditions.
5.10 Discussion

5.10.1 Overview

The low oxygen environment in which placental development occurs in the first trimester of human pregnancy is believed to be important in controlling EVT outgrowth by directing the tightly regulated processes of trophoblast differentiation and invasion (Caniggia et. al., 2000; Genbacev et. al., 1997; Graham et. al., 2000). In this work a two-dimensional model of EVT outgrowth from first trimester villous explants has been used to examine the effects of gestation and oxygen concentration on EVT outgrowth from villi of 8-12 weeks gestation. Unlike three-dimensional models of EVT outgrowth used by some researchers (Aplin et. al., 1999; Genbacev et. al., 1992; Vivovac et. al., 1995), the trophoblast outgrowth in this model is able to be easily quantified allowing the effect of oxygen on EVT outgrowth from first trimester placental villi to be examined in a novel and accurate manner.

5.10.2 The effect of oxygen on the frequency of extravillous trophoblast outgrowth

In this work villi with extravillous columns were not selected as some other researchers do (Aplin et. al., 1999; Caniggia et. al., 2000; Genbacev et. al., 1997), but rather placentae were dissected into small villous explants which were randomly selected for culture. Due to the random nature of selection and the large number of explants cultured from each placenta, a large proportion of the villi from each placenta were used. Thus, there was a good representation of the placenta as a whole. This technique allowed the examination of the number of villous tips in a placenta that were capable of producing EVT outgrowth and thus examine the effect of oxygen on the frequency of EVT outgrowths produced.

Villous explants produced significantly fewer and smaller EVT outgrowths in 1.5% oxygen than in 8% oxygen. These results contrast with those obtained in some other models which have suggested that low oxygen promotes EVT outgrowth (Caniggia et. al., 2000; Genbacev et. al., 1997). Previous reports have shown that in comparison to explants cultured in 20% oxygen, first trimester villous explants of 5 to 8 weeks of
gestation cultured in low oxygen show increased BrdU (thymidine analogue) incorporation, an increase in budding and EVT outgrowth from the tips of villi, as well as an increase in the total number of trophoblasts in this outgrowth (Caniggia et al., 2000; Genbacev et al., 1997). However, the reported ‘increase’ in budding and outgrowth does not appear to have been quantified (Caniggia et al., 2000). Whereas, one of the advantages of the model used in this work is that the size and frequency of EVT outgrowth could be accurately quantified. The difference between the results produced here and those of Caniggia et al. (2000) may also reflect the different gestational ages of the placentae studied as this work has also shown gestational age to be an important determinant of EVT outgrowth (section 3.6), which may not have been appreciated and controlled for in previous work. Alternatively, the differences between the results presented here and those of Caniggia et al. may be explained by their use of physiologically superoxic 20% oxygen (pO₂ = 140mmHg) as a control (Caniggia et al., 2000). Once the maternofetal circulation is established the blood in the IVS is a mixture of arterial and venous blood, and blood sampled from the IVS at term has a pO₂ of 40mmHg (Howard et al., 1961). Therefore, comparative (control) concentrations of around 6-8% oxygen as used in this work represented conditions in vivo once the maternal circulation in the IVS is established more accurately than 20% oxygen. Finally, the differences between the data presented here and that from previously reported results might be explained by the small numbers of explants studied in other reports since there tends to be considerable variation in the behaviour of individual explants. Indeed, a study by Newby et al. in which the authors were unable to draw conclusions on the effect of oxygen on EVT outgrowth from villous explants highlights the difficulties of using a model in which both the nature of the primary tissue used, and the experimental outcomes show a high level of variation (Newby et al., 2005). However, such variability does not detract from the accuracy of explant culture studies, but rather when large numbers of villi are used to overcome the variation inherent to the culture system, explant culture allows the natural variation that is likely to occur between villi in vivo to be demonstrated in vitro. In this work, a substantially larger number of explants (3693) and placentae (25) have been investigated than other studies to date, thereby allowing an accurate representation of villi from the whole placenta over a range of gestational ages.
5.10.3 Effect of oxygen on the area of extravillous trophoblast outgrowth

In this work, villous explants were cultured on an extremely thin layer of Matrigel, which results in two-dimensional EVT outgrowth that was readily and accurately quantifiable. This model provided the advantage that small but significant changes in outgrowth and individual trophoblast area are able to be quantified in a way that is not possible in the majority of other models. Analysis of outgrowths demonstrated that those from villous explants cultured in 1.5% oxygen had a significantly smaller area than those from villous explants cultured in 8% oxygen. Further investigation revealed that individual EVTs in outgrowths had a smaller mean area when cultured in 1.5% oxygen than in 8% oxygen. EVTs proximal to the villi were smaller than those in the distal regions of the outgrowth. The increased area of trophoblasts in the distal columns is likely to be due to the increase in available space as trophoblasts migrate away from the villus and spread across the Matrigel surface. However, the observed difference in individual EVT area between oxygen conditions was irrespective of the location of cells within the outgrowth (i.e. the effect was observed in both the proximal and distal regions of the outgrowth). This effect has not been reported previously, and it is unclear how oxygen concentration would affect cell size. One possibility is that the measured two-dimensional area may be reduced by rounding of the cells on the culture plate, thereby not representing a true change in overall cell volume but rather an effect on the adhesiveness of EVTs to the Matrigel or a decrease in cell spreading. In vivo a decrease in trophoblast adhesiveness in 1.5% oxygen could potentially assist in the process of trophoblast invasion by aiding EVT migration. It was not possible to determine accurately if the decrease in cell size was a result of a rounding of the EVTs from the data collected, however it would be of future interest to study the effect of oxygen on EVT volume. A second possibility is that in many cell types low oxygen concentrations are detrimental to the cell’s ability to function successfully and undertake protein synthesis, which may result in apoptotic death (Jain & Sznajder, 2005; Slater et. al., 1995). Thus, a decrease cell size in such cases may be due to a reduction in function or condensation in preparation for apoptosis (Bortner, 2005). However, as the placenta normally develops in low oxygen conditions in the first trimester, trophoblasts from early first trimester placentae are uniquely equipped to grow in low oxygen conditions, as shown by the rapid growth of the placenta over the first trimester and the level of villous trophoblast proliferation. Furthermore, EVT outgrowth is viable in both 1.5% and 8% oxygen conditions, as shown by Trypan Blue exclusion. Therefore, in first trimester placental tissue, it seems
unlikely that the observed decrease in EVT area in 1.5% oxygen would be primarily a result of declining cell viability as may be expected in other cell types. It remains unclear what the functional significance of a decrease in EVT size in the low oxygen conditions which exist for the majority of the first trimester would be in vivo.

The reduced area of individual EVTs partly explains the reduced size of EVT outgrowths cultured in 1.5% oxygen, but cannot completely explain the 32% reduction in EVT outgrowth area. Individual EVTs cultured in 1.5% oxygen were only 23.4% smaller than those cultured in 8% oxygen. Therefore, the decrease in individual trophoblast area in 1.5% oxygen accounts for only 2/3 of the difference in total outgrowth area, and a reduced number of trophoblasts in the outgrowths must also have contributed significantly to the overall reduction in EVT outgrowth.

5.10.4 Possible explanations for the observed effects of oxygen on extravillous trophoblast outgrowth

Over and above the decrease in the area of individual EVTs, the decrease in overall EVT outgrowth area and frequency in 1.5% oxygen compared to 8% oxygen could arise from several different scenarios:

1) Only the EVTs proximal to villi proliferate and are able to drive outgrowth expansion (Caniggia et. al., 1997; Genbacev et. al., 1992; Vivovac et. al., 1995).
   Low oxygen conditions may inhibit the proliferation of EVTs in the proximal columns, thereby reducing the pool of trophoblasts able to form EVT outgrowth and the rate of outgrowth expansion.

2) It is possible that 1.5% oxygen conditions favor proliferation of the progenitors of EVT in the villi over migration of those cells into EVT columns such that there is a reduced migration of EVTs from the villi.

3) The smaller area of outgrowth observed in 1.5% oxygen conditions may represent a greater proportion of villous explants that did not produce outgrowth until later in the culture period.

At present using this model it has not been possible to distinguish between these three potential explanations.
Taken together, the large number of villous explants and outgrowths studied shows a clear decrease in frequency and area of EVT outgrowth in 1.5% oxygen in comparison to 8% oxygen. This confirms that cytotrophoblast differentiation into EVT is regulated by oxygen. The decrease in EVT area further shows that individual EVTs are also regulated by oxygen.

5.10.5 Interdependent effects of gestational age and oxygen on extravillous trophoblast outgrowth from first trimester villi

It is commonly believed that the trophoblast plugs which occlude the maternal spiral arteries gradually disperse from 10 weeks of gestation, allowing maternal blood to flow into the IVS inducing a rise in oxygen tension across the villous surface (Jaffe & Woods, 1993; Jauniaux et al., 2003b; Jauniaux et al., 1992). It was interesting to note that when the differential response of villous explants from the same placenta to 1.5% or 8% oxygen was examined there was an effect of oxygen concentration only for those placentae of less than 11 weeks of gestation. It was shown previously (section 3.6) that gestation independently affects the frequency of EVT outgrowth. Thus, by each placenta acting as its own gestation-matched control the effect of oxygen by gestational age was able to be examined without confounding the results with the previously determined effect of gestation on EVT frequency in the first trimester. It was anticipated there would be a gradual decline in the effect of oxygen concentration as gestational age increased from 8 to 12 weeks of gestation as the trophoblast plugs dissipate slowly and the placenta becomes exposed to increasing oxygen concentrations in vivo (Jauniaux et al., 2003b; Jauniaux et al., 1992). However, a sharp change in the responsiveness to oxygen was observed, with a clear lack of responsiveness of outgrowth production to low oxygen in placentae of 11 weeks of gestation and greater. This result suggests that there is a shift from a low oxygen environment around 10 weeks of gestation such that the lack of responsiveness after this time may be either a result of the placenta preparing itself for the entry of maternal arterial blood into the IVS, or a response to increasing oxygen concentration as the trophoblast plugs dissipate. Indeed, the antioxidant superoxide dismutase enzymes are not expressed in the placenta until 8-9 weeks of gestation, and conversely villous HIF-1α expression decreases at around 9 weeks of gestation and is absent by 12 weeks of gestation (Caniggia et al., 2000; Caniggia & Winter, 2002; Watson et al., 1997; Watson et al., 1998). Therefore, the lack of responsiveness of villous
explants from placentae of 11 and 12 weeks of gestation may be because placentae of these gestations have already gained the ability to adapt to increased oxygen concentrations \textit{in vivo}. This data supports the growing body of evidence that the trophoblast plugs dissipate exposing the placenta to maternal blood and increased oxygen concentrations from approximately 10 weeks of gestation, but suggests that there may be a sharp, rather than a gradual change, to arterial-like oxygen concentration in the IVS between 10-11 weeks (Jaffe \textit{et. al.}, 1997; Jauniaux \textit{et. al.}, 2003b; Jauniaux \textit{et. al.}, 1992; Jauniaux \textit{et. al.}, 2001; Rodesch \textit{et. al.}, 1992)

5.10.6 The effect of oxygen on extravillous trophoblast phenotype

The expression of three different markers of EVT differentiation – HLA-G, $\alpha$1 integrin and p27 - were used to examine the effect of oxygen concentration on EVT phenotype in outgrowths from first trimester villi. \textit{In vivo}, HLA-G expression is upregulated as EVTs migrate away from the villi in proximal columns (McMaster \textit{et. al.}, 1995; Shorter \textit{et. al.}, 1993), and a previous study has reported a decrease in the expression of HLA-G in 2% oxygen in comparison to 20% oxygen in the transformed trophoblast cell line HTR-8/SVneo (Kilburn \textit{et. al.}, 2000). However, in contrast to those previous studies in this work it was observed that EVTs stained strongly for HLA-G throughout the cell columns in both 1.5% and 8% oxygen. Staining of HLA-G throughout EVT outgrowth was also demonstrated in 20% oxygen (section 3.2.2). In support of this data, more physiologically relevant experiments than those of Kilburn \textit{et. al.} on outgrowth from explants and isolated first trimester trophoblasts showed no change in the expression of either the membrane bound or soluble isoforms of HLA-G between 20% and 2% oxygen (Genbacev \textit{et. al.}, 1997; Nagamatsu \textit{et. al.}, 2004b). Furthermore, observations of placental bed biopsies show that HLA-G expression changes only in the proximal EVT columns (Bhalla \textit{et. al.}, 2006). Once EVTs enter the decidua HLA-G is not further upregulated, nor is there an apparent difference in the level of HLA-G expression between endovascular trophoblasts which are exposed to arterial oxygen levels and EVTs in the surrounding decidua, which are exposed to lower oxygen levels, suggesting that HLA-G expression is not regulated by oxygen concentration (Bhalla \textit{et. al.}, 2006).

EVT expression of $\alpha$1 integrin is believed to be involved in facilitating trophoblast invasion into the maternal decidua as it complexes with $\beta$1 integrin to form a receptor
for laminin and collagen. $\alpha_1$ integrin expression is observed in distal regions of EVT outgrowth in vivo, and is essential for trophoblast invasion (Damsky et. al., 1992; Damsky et. al., 1994). In contrast to previous reports that $\alpha_1$ integrin expression in EVT outgrowths was greater in 20% oxygen than in 2% oxygen (Genbacev et. al., 1997), in this work $\alpha_1$ integrin, like HLA-G, was expressed throughout the cell columns of all EVT outgrowths with no difference in expression observed between 1.5% and 8% oxygen.

The gradient of HLA-G and $\alpha_1$ integrin expression previously reported in EVT columns in vivo has been at least partly attributed to an increasing oxygen gradient as the EVTs invade into the maternal decidua in vivo (Genbacev et. al., 1997). However, in this work both HLA-G and $\alpha_1$ integrin were expressed throughout EVT outgrowths in both 8% and 1.5% oxygen. In this in vitro situation, the entire trophoblast column length is exposed to either 1.5% or 8% oxygen, whereas in vivo there may be an oxygen gradient along the length of the EVT columns. This could potentially explain why there is no difference along the length of the column in vitro, but cannot explain why there was no difference between the oxygen conditions. These results indicate that these molecules are not regulated by oxygen concentration and rather that their increased expression by EVTs as they migrate away from villi in vivo is a feature of their invasive phenotype and is induced by other factors. It is possible that the uniform expression of both HLA-G and $\alpha_1$ integrin throughout EVT outgrowth in vitro is a result of the absence of additional regulatory molecules in the explant model that may have potentially been derived in vivo from either the decidua or the villous mesenchyme (which is not viable by 48 hours in this culture model as shown in chapter 3). A further possibility is that the use of Matrigel may have the potential to affect the expression of $\alpha_1$ integrin and HLA-G. As $\alpha_1$ integrin complexes with $\beta_1$ integrin to form the collagen receptor, the contact of EVTs with the collagen present in Matrigel may induce $\alpha_1$ integrin expression. However, this is unlikely as previous reports showing an effect of oxygen on $\alpha_1$ integrin expression also employ three-dimensional Matrigel explant models (Genbacev et. al., 1997).

Furthermore, culture of HTR-8/SVneo cells on Matrigel induced the expression of HLA-G in comparison to culture on individual ECM proteins (Kilburn et. al., 2000). However, as culture of HTR-8/SVneo cells on Matrigel did not affect the ability of HTR-8/SVneo cells to downregulate HLA-G expression in low oxygen conditions, it is again unlikely to affect the results in this work (Kilburn et. al., 2000).
Another set of markers by which EVT differentiation can be examined are cell cycle regulators. In this work the expression of the cell cycle inhibitor p27 and its response to oxygen concentration was examined in EVT outgrowths cultured in 1.5% or 8% oxygen. Only a few studies have previously examined the role of p27 in EVT differentiation. These studies report that in vivo only EVT cells which have invaded deep into the decidua express p27 (Fukunaga, 2004; Korgun et al., 2006). Of those EVT outgrowths in this thesis that did express p27, expression was only evident in infrequent nuclei on the periphery of the outgrowths, and no difference was observed between p27 expression by EVTs between 1.5% and 8% oxygen. Only one previous study has addressed the regulation of p27 by oxygen in trophoblast (Ma et al., 2001). Ma and colleagues found that culture of the ED27 trophoblast cell line in 2% oxygen resulted in weak p27 expression whereas, when the same cells were cultured in 20% oxygen, p27 was upregulated (Ma et al., 2001). However, these authors examined p27 expression by western blotting, and were therefore not able to determine whether p27 expression was nuclear or cytoplasmic. p27 acts by binding to nuclear cyclins which are involved in facilitating the cell cycle, thereby inhibiting their actions (Hall et al., 1995). Therefore, only nuclear expression of p27, as shown in the EVTs in this chapter, is important functionally. It is interesting that the expression of this cell cycle inhibitor was restricted to the periphery of the EVT outgrowth when it has been shown in this thesis that EVT outgrowths in this model do not actively proliferate in any region of the outgrowth visible on the Matrigel surface, as evidenced by the absence of Ki67 expression (section 3.3). It is also unclear why less than 50% of EVT outgrowths showed any expression of p27. p27 expression did not appear to be related to the size of the outgrowth. However, the size of the EVT outgrowth does not necessarily correspond to the age of outgrowth in this model, and the progression of EVT outgrowth was not able to be monitored in this culture system. Therefore, it is possible that the expression of p27 may have been related to the age of individual EVTs.

Taken together, these results suggest that the expression of HLA-G, α1 integrin and p27 are not regulated by oxygen and thus these molecules are not markers of responses to changing oxygen in trophoblasts.
5.10.7 Summary

It has been shown that culture of villous explants in low oxygen results in a decrease in the frequency and size of EVT outgrowths from first trimester villous explants. Furthermore, there is a difference in the ability of villous explants to produce EVT outgrowth and respond to changes in oxygen concentration with gestational age. These results also question whether HLA-G, α1 integrin and p27 are regulated by oxygen in the placenta in the first trimester. The data presented here clearly shows that both gestational age and oxygen play important regulatory roles in EVT outgrowth during the first trimester in human pregnancy.
Chapter Six:

Concluding Discussion
6.1 Why study cytotrophoblast differentiation?

Many pregnancy-related complications that present late in gestation appear to have their origins early in pregnancy with abnormalities in implantation and placental development. Therefore, the differentiation of cytotrophoblasts down the EVT lineage in the first trimester of pregnancy is not only vital for the success of implantation, but for the success of the pregnancy right through gestation. In the first trimester, EVTs grow out from the placenta and invade into the maternal decidua, acting to both physically attach the placenta to the decidua, and to modify the maternal spiral arteries to into large bore conduits capable of providing the fetus with an adequate supply of blood and nutrients as gestation progresses. Inadequate EVT invasion, has been implicated in the pathophysiology of such conditions as miscarriage, pre-eclampsia and intra-uterine growth restriction (Ball et. al., 2006; Brosens et. al., 1972). Pre-eclampsia affects 2-3% of pregnancies, and is a leading cause of maternal and neonatal death worldwide (Goodburn & Campbell, 2001). The extent of EVT invasion in pre-eclampsia is frequently shallow and the extent of spiral artery remodeling is reduced (Brosens et. al., 1972). Furthermore, a number of factors known to regulate trophoblast differentiation have been found to be abnormal in pre-eclampsia (Caniggia et. al., 2000a; Chen et. al., 2004; Lee et. al., 2001). This suggests that EVT differentiation plays an important role in the development of pre-eclampsia.

Spontaneous miscarriage is reasonably common, affecting approximately 12 to 15% of clinically recognised pregnancies. Much less common is recurrent miscarriage, defined as 3 or more consecutive first trimester miscarriages (Stirrat, 1990). The theoretical chance of having three consecutive miscarriages based on a spontaneous miscarriage rate of 15% is only 0.34%. However, the actual rate of women affected by recurrent miscarriage is approximately 1%, which is greater than expected by chance alone. Therefore, other factors must be contributing to the increased rate of miscarriage in these women (Stirrat, 1990). Recurrent miscarriage has been associated with abnormal trophoblast differentiation early in pregnancy, including a decrease in cytotrophoblast proliferation, a decreased rate of cytotrophoblast fusion into syncytiotrophoblast and a decreased area of trophoblast in placental villi, and this has been suggested to be linked to a premature onset of maternal perfusion (Bose et. al., 2006). Indeed, premature exposure of the
placenta to increased oxygen concentrations has been associated with miscarriage (Jauniaux et al., 2003b).

It is clear that cytotrophoblast differentiation down the EVT lineage, which is the key prerequisite for the processes of EVT outgrowth, invasion, and spiral artery transformation, is an important factor in determining pregnancy outcome. However, the factors controlling cytotrophoblast differentiation in normal pregnancy, let alone abnormal pregnancy, are poorly understood. The work in this thesis has contributed to the understanding of cytotrophoblast differentiation in three ways:

1) It has developed two novel models by which cytotrophoblast differentiation is able to be studied.

2) It has identified a sub-population of EVT progenitors which are likely to be committed to differentiation into EVTs. This will allow the correct cytotrophoblast population to be studied in order to accurately understand cytotrophoblast differentiation along the EVT lineage.

3) It has quantified for the first time the effect that both oxygen and gestational age have on EVT outgrowth from first trimester villi.

6.2 The importance of gestational age within the first trimester

First trimester placentae are often assumed to behave in the same manner, regardless of their gestational age. However, a substantial amount of growth and differentiation occurs within the placenta during the first trimester, and it is becoming evident that different cell types in the placenta do not behave consistently throughout the first 12 weeks of gestation. A recurrent theme throughout this thesis was that the gestational age of the tissue being examined influenced the results obtained, and the label ‘first trimester’ is not an appropriate description by which to group placentae when studying the behaviour of trophoblast. Differences in the behaviour of placentae by gestation within the first trimester have been previously noted in the literature. In vivo, there is a high level of mitoses in the villous cytotrophoblast between 6 and 10 weeks of gestation, but the mitotic index decreases significantly between 10 and 12 weeks of gestation when the placenta is exposed to maternal blood (Tedde & Tedde-Piras, 1978). Furthermore, Aboyage-Mathiesen et al. saw a difference in the populations of cytrotrophoblasts they were able to obtain from placentae of 10 weeks of gestation and under (Aboagye-
Mathiesen et al., 1996). Differences in placental behaviour with gestational age have also been observed in this thesis. In chapter 3 it was shown that the proportion of villi with the potential to produce EVT outgrowth decline with increasing gestational age between 8 and 12 weeks of gestation, and a striking difference in the response of villous explants to low oxygen conditions in chapter 5 was observed at 11 weeks of gestation. The combination of the above factors resulted in the decision to use only placentae of less than 10 weeks of gestation for the trypsinization of 10 day old explants in chapter 4, and indeed it was noted that the most successful of these cultures were from the younger placentae used. On the rare occasions when trypsinization of placentae greater than 10 weeks of gestation was attempted these cultures were not successful. Finally, the importance of the effect of gestation on EVT differentiation also has implications for future placental research, and suggests that for the study of EVT differentiation it may be advantageous to use placentae of younger gestations to obtain clearly defined and accurate results. Conversely, it has been shown that the study of syncytiotrophoblast shedding benefits from the use of older placentae (Abumaree et al., 2006; Huppertz et al., 2003).

6.3 Trophoblast stem cells

Stem cells are capable of proliferation, self renewal, and conversion to differentiated cells. There are two main types of stem cells – embryonic stem cells, which are pluripotent and capable of differentiating into any cell type, or ‘adult’ (non-embryonic) stem cells, which are multipotent and more limited in their capacity to differentiate, usually only being capable of differentiating into the mature cell types of the tissue in which they reside. Once a cell is terminally differentiated it is generally considered to be committed to its final form and function, and has limited regenerative capacity. Embryonic stem cells arise from the inner cell mass of the blastocyst or from pre-morulla embryos, and their differentiation is able to be directed by specific culture conditions, although this is often a poorly defined, inefficient and relatively non-selective process resulting in a heterogenous population of differentiated and undifferentiated cells. Human embryonic stem cells have been able to be manipulated to obtain human cytotrophoblast stem cell lines that show bipotential differentiation into either cells characteristic of syncytiotrophoblast, or into an invasive phenotype characteristic of EVT's (Harun et al., 2006). In vivo the trophoblast cell lineage is considered to arise at the
blastocyst stage from the trophoectoderm that surrounds the inner cell mass, and contributes exclusively to the extraembryonic structures, and at this stage of development a self-renewing trophoblast stem cell population is evident in both rodents and humans (Benirschke & Kaufmann, 2000; Tanaka et al., 1998). However, a true trophoblast stem cell population remains to be identified. The evidence in this thesis suggests that by approximately 7 weeks of gestation, villous cytotrophoblasts are no longer bipotent stem cells, but are committed to differentiation down either the EVT of syncytiotrophoblast lineage. Therefore, the question remains as to what truly constitutes the human trophoblast stem cell population, and at what stage of development is it possible to obtain a population of truly uncommitted stem cells? Little information is currently available by which to answer these questions, and it remains unclear whether the cytotrophoblast buds that protrude into the primitive syncytiotrophoblast lineage during the initial stages of villous formation are bipotent or potentially multi-potent progenitors at this time, or whether even at that very early (12 days post fertilization) stage two separate populations of cytotrophoblast progenitors (EVT and syncytiotrophoblast) exist. It may also be possible that a limited population of adult stem cells may be present in mature placental villi even though the vast majority of cytotrophoblasts appear to be committed to either EVT differentiation or syncytiotrophoblast formation at this time. Indeed, ‘side populations’ of multipotent adult stem cells have been isolated from a range of mammalian tissues that show low staining with Hoescht dye when examined by flow cytometry due to their ability to efflux this dye (Challen & Little, 2006). Understanding the true nature trophoblast stem cells will require novel approaches by which to investigate the events of very early implantation.

Much has been learned about growth factor and cytokine signaling in differentiated cell types, however because stem cells are few in number and typically cannot be expanded substantially in culture substantially less is known about the mechanisms controlling their survival, proliferation and differentiation. It is possible to culture mouse trophoblast stem cells that are capable of differentiating into all three mouse trophoblast lineages (Cross et al., 2003; Tanaka et al., 1998). Thus there is a substantial body of work on the genes and signaling pathways involved in directing mouse trophoblast differentiation. However, the development of the human placenta and the extent of EVT outgrowth and invasion that occurs in human pregnancy differ from that of the mouse, and a culture
model by which human stem cells could be studied in this depth would have significant advantages. At this stage however, the limited time use of human primary villous cytotrophoblasts after isolation and the lack of a clearly identified proliferative, stem cell-like population has significantly limited the ability to genetically manipulate and analyze the mechanisms involved in the regulation of trophoblast stem cell differentiation, the commitment of stem cells to cell lineages and the signaling mechanisms involved in these processes.

6.4 Future Directions

The identification of a novel sub-population of EVT progenitors has shed new light on one of the most fundamental and important processes involved in implantation – the differentiation of cytotrophoblast into EVT, which are fundamentally responsible for so many of the vital interactions between the mother and baby. Thus, while the identification and initial characterisation of this sub-population in this thesis provides an important step forward in the understanding of EVT outgrowth formation, in many ways it also opens up the field of trophoblast differentiation by creating an entirely new way of considering cytotrophoblast differentiation.

The isolation of a population of cytotrophoblast stem cells which are able to proliferate in culture and differentiate into EVTs raises the enticing possibility of creating a pure EVT progenitor cell line. During the course of this work numerous unsuccessful attempts were made to accomplish this, and several large obstacles remain to be resolved before the continuous culture of EVT progenitors as a cell line will be able to be made a reality. Firstly, after the initial burst of proliferation observed in the first few days of culture the population doubling time of these cells proved to be very slow. This may be due to the fact that they are extremely sensitive to the culture conditions employed. Conditioned media was essential to the success of these cultures, and changes in the conditioned media or the batch of Matrigel both proved detrimental to EVT progenitor survival at times during this work. Purifying the trophoblasts from the low levels of contaminating fibroblasts also proved to be difficult as the cells did not proliferate when plated at low dilutions (15 cells per well) and as the EVT progenitors expressed CD9, antibody depletion using this common fibroblast marker was not possible. Therefore the key obstacles which will need to be resolved in order to obtain a regenerating cell line
from this sub-population in the future are firstly the identification of a marker by which these cells can successfully be selected for, but more importantly discovering the specific factors which promote their survival and enable the *in vitro* recreation of the specific microenvironment present in the villous tips in which they show remarkable survival *in vivo*.

In this work the markers used to examine whether the isolated cytotrophoblasts were characteristic of the multilayered cytotrophoblasts present in the cell islands of villous tips *in vivo*, which have been hypothesized to be EVT progenitors, were all taken from previous reports in the literature. However, it would be of great value to develop this methodology further in order to obtain a pure trophoblast population by which proteomic or microarray technology could be used to look for novel markers of this population in comparison to monolayer villous cytotrophoblasts that are predominantly obtained in isolates from fresh first trimester placentae.

Other questions raised by this work which should be addressed in the future are:

- Are putative EVT progenitors able to be cloned and propagated?
- How would putative EVT progenitors respond to being cultured on tenascin or other ECM compounds?
- What factors affect the differentiation of EVT progenitors? Is it possible to induce differentiation down the syncytial pathway or are they truly committed to the EVT pathway?
- Do EVT progenitors exhibit the characteristic Hoechst stain side-population profile often associated with stem cell populations?
- Why do villous explants produce fewer outgrowths that contain less cells in low oxygen conditions and how does oxygen act on EVT progenitors in the tips of explants, or isolated *in vitro*?
- How does oxygen affect EVT progenitors?
Appendix A:

MMP Activity in Placental Explants and an Evaluation of Gelatin Zymography as a Quantitative Method for Assessing MMP Activity
A.1 Introduction and rationale

Matrix metalloproteases (MMP) -2 and MMP-9 are expressed by the villous placenta and EVT in the first trimester of pregnancy and function to facilitate EVT invasion by digesting components of the ECM. MMP-2 and MMP-9 are expressed in a pro-enzyme form which is activated in the extracellular compartment. Therefore, total amounts of MMPs determined by immunohistochemistry or western blotting may not be an accurate representation of the activity of these proteins. However, gelatin zymography provides a useful method by which MMP activity can be determined. Zymography is a technique which has been used to analyze the activities of matrix metalloproteinases (MMPs) in complex biological samples (Canning et. al., 2001; Niu et. al., 2000; Riley et. al., 2000). This technique involves two major steps. Firstly, the proteins are separated by electrophoresis under denaturing but non reducing conditions in a gelatin containing polyacrylamide gel. Secondly, the resolved proteins are renatured by exchange of the SDS with a non-ionic detergent (such as Triton-X100), thereby allowing them to digest the gelatin. This results in zones of clearance that represent proteolytic activity when the gel is stained with coomassie blue. Zymography has previously been reported as a quantitative methodology for MMP activity (Canning et. al., 2001; Niu et. al., 2000; Riley et. al., 2000). The level of activity in each band on a zymography gel can be determined by measuring its optical density. As the optical density of each band produced depends both on the amount of protein present in the band and the biological activity of the enzyme in the band, it is important to standardize methods and determine inter-gel accuracy and consistency in the results produced.

A.2 Consistency of zymography results between gels

In order to be able to quantify differences between conditions and analyze a large number of samples by gelatin zymography, results must be accurate and consistent between gels. In order to test the consistency of zymography, 6 different pooled conditioned media samples from placental explant cultures were run on two zymography gels under identical conditions (Figure A.1). The corresponding bands on each gel appeared visually similar, however more detailed analysis using Quantity One software to calculate band volumes revealed significant differences in the band volume of identical samples between the two gels (Table A.1). This inter-gel variability reveals a significant problem in this technique as it provides an unacceptable amount of variation between
identical samples. The average standard error of the band volume for each identical sample across the two gels was 0.107OD/mm², which represents 19.7% of the average band volume, and 17.9% of the range in the data. Furthermore, an additional standard error of 0.04OD/mm² is generated in the process of optical density measurement. Therefore, this level of error between gels shows that gelatin zymography is not accurate enough to determine differences in MMP activity at the sensitivity required for media samples from this explant model.

Figure A.1 – Two identical gelatin zymograms demonstrate that results obtained from running identical pooled explant media samples are visually difficult to distinguish. The blue gelatin-containing background stains strongly with coomassie blue, whereas the areas of MMP activity where the gelatin has been digested appear as clear bands. However, quantitative analysis of the main band in the samples (MMP2) reveals significant differences in the intensity of the bands (refer Table A.1 below).
### Table A.1 – Table demonstrating the large quantitative variations in band volumes between media samples run on matched zymography gels (refer Figure A.1). Values represent averages of triplicate measurements of the optical density of band volume for each gel. This table shows that a) the measurable differences between samples are not much greater than the variation in data between gels and b) due to the large amount of standard error, samples can not be significantly differentiated from each other in terms of band volume. Band volumes were calculated using BioRad Quantity One software, and provide a quantitative method of measuring the activity of the enzymes in each band by the amount of background gelatin digested. Global background values were set against a large selection of gel below the digested samples in Figure A.1 (the area used to set the background has been cropped out of the image). Media samples are described by their gestational age and production of outgrowth, “OG” = Pooled media from explants that produced outgrowth in an individual placental culture, whereas “no OG” = pooled media from explants that did not produce outgrowth in an individual placental culture.

<table>
<thead>
<tr>
<th>Media Sample</th>
<th>Gel A</th>
<th>Gel B</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Average band volume, adjusted for background (OD/mm²)</td>
<td>Average band volume, adjusted for background (OD/mm²)</td>
</tr>
<tr>
<td>1: 11.4wks, OG</td>
<td>0.888 (±0.044)</td>
<td>0.352 (±0.026)</td>
</tr>
<tr>
<td>2: 11.4wks, no OG</td>
<td>0.375 (±0.034)</td>
<td>0.449 (±0.032)</td>
</tr>
<tr>
<td>3: 10.3wks, OG</td>
<td>0.584 (±0.099)</td>
<td>0.504 (±0.004)</td>
</tr>
<tr>
<td>4: 10.3wks, no OG</td>
<td>0.479 (±0.026)</td>
<td>0.289 (±0.011)</td>
</tr>
<tr>
<td>5: 10.2wks, OG</td>
<td>0.773 (±0.066)</td>
<td>0.415 (±0.027)</td>
</tr>
<tr>
<td>6: 10.2wks, no OG</td>
<td>0.738 (±0.035)</td>
<td>0.680 (±0.0173)</td>
</tr>
</tbody>
</table>

In order to confirm the discrepancies in band volume demonstrated between the gels above, the same dilution of a collagenase positive control was measured in 13 different gels. The mean optical density of the clearance zones digested by the collagenase in each of the 13 zymograms was 3.22OD/mm². However, a large level of inter-gel variation was again seen as the standard deviation of this data was 1.98, and the standard error of this data was 0.55OD/mm² (17.1%).
A.3 The generation of a standard curve of zymography activity

The large amount of error in samples split between gels meant that differences in MMP activity between zymograms would not be able to be accurately determined without careful standardization. Therefore, a series of diluted standard samples would need to be run on each zymogram in order to accurately compare results between individual zymograms. In order to first assess the accuracy of creating a standard curve of collagenase dilutions, collagenase stock was diluted 1:100, 1:200, 1:400, and 1:800 and run on duplicate zymograms to create a standard curve. The standard curve from each zymogram was created by determining the average optical density from triplicate measurements of the main collagenase band at each dilution (Figure A.2). These results show that an accurate standard curve can be created by gelatin zymography over large changes in concentration. However, of the duplicate standard curve gels run, Zymogram 1 produced a good fit for a linear model ($R^2 = 0.9923$), but a poor fit for a logarithmic model ($R^2 = 0.8663$), whereas Zymogram 2 produced a poor fit for a linear model ($R^2 = 0.8937$), and a good fit for a logarithmic model ($R^2 = 0.9955$). Therefore it is unclear which model would be accurate, and this further demonstrates the unreliability of the assay. In addition, the difference in raw data from the duplicate standard curves created again demonstrates the potential for large variability between gels. Therefore, a standard curve would need to be run on every zymogram of unknowns, meaning that a substantial proportion of each zymogram would consist of standards, rather than unknowns. This means that gelatin zymography is not able to be used as an accurately quantitative method of measuring MMP activity in this model. However, gelatin zymography may still be used to demonstrate MMP activity in a qualitative manner.
Figure A.2 – Graph demonstrating the differences in the standard curves created from identical samples of collagenase run on separate zymography gels. Values represent averages of triplicate measurements of the optical density of clearance zone band volume for each zymogram after background subtraction. There is considerable variation between the standard curves generated. Furthermore, the two gels show different patterns of best fit – with Zymogram 1 showing a linear trend (as graphed), whereas Zymogram 2 shows a logarithmic trend.

A.4 Expression of matrix metalloproteases in villous explant culture

While the above data shows that it is not possible to determine the relative amounts of MMPs secreted under different conditions, zymography analysis still allows the visualisation of active MMP-2 and MMP-9 expression. In order to examine the secretion of MMPs from the villi, supernatants from 84 villous explants were examined by gelatin zymography, which identifies only the active form of the proteins. It was found that both MMP2 and MMP9 were secreted by all explants regardless of whether they had produced EVT outgrowth (n=42) or not (n=42) (Figure 3.7). Examination of supernatants from 36 explants cultured in 1.5% oxygen or 48 explants cultured in 8% oxygen revealed that both MMP-2 and MMP-9 were secreted by villi cultured in both oxygen conditions.
Figure A.3 – Gelatin zymogram (digitally inverted) demonstrating the activity of MMP9 and MMP2 in two samples of media from individual villous explants from an 8.6wk placenta cultured in 8% oxygen (dark bands). The left hand lane contains marker of standard molecular weights (white bands).

A.5 The importance of villous explant MMP expression

The ability of trophoblasts to invade into the decidua in the first trimester is essential for the success of pregnancy, and the MMP system is a key mechanism by which EVTs are able to facilitate invasion (Bischof et. al., 2002; Seval et. al., 2004). In this work MMP expression was observed in media from both explants that did and explants that did not produce EVT outgrowth. Therefore, MMP production must arise at least in part from the villi. The exact source(s) of villus MMP production in this model was not clear as MMP activity measured in the cell culture media may have been derived from any of the cells in the villi, and MMP expression has been reported in the syncytiotrophoblast, cytotrophoblasts, and mesenchymal cells (Autio-Harmainen et. al., 1992; Niu et. al., 2000; Staun-Ram et. al., 2004). In addition, MMP activity was evident in lysates of EVTs purified from explant culture. It is therefore interesting to note that even though the EVTs do not need to digest the Matrigel to migrate from the villous explant, they still express significant levels of MMP-2 and MMP-9, the gelatinases secreted by trophoblasts, supposedly in order to break down the ECM and facilitate invasion. However, the differences between MMP production in media from explants that did and explants that did not produce EVT outgrowth was not able to be quantified as the zymography system used was not deemed to be able to accurately quantify enzyme activity (section A.3)
The results in this thesis showed predominant activity of MMP-2 over MMP-9 in conditioned media and EVT lysates from explants from placentae of both 8 and 12 weeks of gestation. There are conflicting reports regarding MMP expression and activity in the first trimester, with evidence for both predominant activity of MMP-2 (Seval et. al., 2004) and conversely for predominant activity of MMP-9 (Peters et. al., 1999; Shimonovitz et. al., 1994). This conflicting data may have arisen from the range of cell lines and isolated primary cell preparations used, as differing MMP expression profiles have been observed in cytotrophoblasts, syncytiotrophoblast, EVTs and mesenchymal cells (Isaka et. al., 2003; Niu et. al., 2000; Sawicki et. al., 2000). Furthermore, a gestational effect of MMP-2 and MMP-9 activity in isolated trophoblasts has previously been reported with trophoblasts from placentae of 6-8 weeks of gestation showing dominant expression of proMMP-2 and proMMP-9, whereas trophoblasts from placentae of 9-12 weeks of gestation proMMP-9 expression was dominant over proMMP-2 expression (Staun-Ram et. al., 2004; Xu et. al., 2000). In contrast, the data in this thesis showed a predominance of MMP-2 over MMP-9 in conditioned explant media and EVT cell lysates from both 8 and 12 weeks of gestation. The differences between the results presented here and reports in the literature may be due to several reasons:

1) Staun-Ram et. al. (2004) measured proMMPs by gelatin zymography, however as discussed above, gelatin zymography is not accurately quantitative (Staun-Ram et. al., 2004).

2) Staun-Ram et. al., (2004) measured MMP activity in isolated cytotrophoblast cells, which are not a naturally invasive trophoblast population in vivo (Staun-Ram et. al., 2004). Therefore, measurement in pure, isolated, EVTs and in media from intact explants provides a more relevant model of MMP expression. Indeed, in concordance with the results in this thesis, in a study using intact explants Niu et. al. (2000) showed a predominance of MMP-2 (in both pro and active forms), which did not occur in their cultures of isolated cytotrophoblasts (Niu et. al., 2000).

3) The predominance of MMP-2 in conditioned media from explant cultures, in comparison to the predominance of MMP-9 secretion from isolated cytotrophoblasts, indicates that MMP-2 may be secreted abundantly from cells other than cytotrophoblasts in intact explants (Niu et. al., 2000). Indeed, MMP-2 has been reported to be produced by placental fibroblasts, syncytiotrophoblast,
and to be predominant in EVTs (Autio-Harmainen et. al., 1992; Isaka et. al., 2003; Petignat et. al., 2006; Polette et. al., 1994; Vegh et. al., 1999).

4) Alternatively it is possible that the fibroblasts stimulate MMP-2 production by cytotrophoblasts and/or EVTs in models where intact villi are used. Both MMP-2 and MMP-9 have been shown to be important for trophoblast invasion (Bischof et. al., 1995).

Thus, that the findings regarding MMP-2 predominance and a lack of effect of gestational age in this thesis differ from the majority of previous data, demonstrate how the intercellular interactions that are maintained in explant models can result in different, more accurate, findings than those from isolated cell culture.

Zymography permits detection of latent and active forms of MMPs because exposure to SDS results in a conformational change which results in activation and hence both the activity of pro and mature forms of MMPs are able to be visualised. Therefore, it is interesting that unlike the majority of other authors who report detection of the pro-enzyme forms of MMP-2 and 9, the molecular weights of the bands of activity observed in this thesis corresponded to the mature forms of MMP-2 and MMP-9 (Niu et. al., 2000; Staun-Ram et. al., 2004; Xu et. al., 2000). However, instances of an active form of MMP-2 and MMP-9 have been reported, in conditioned media from organ culture of first trimester placentae (Niu et. al., 2000). One explanation for an absence of pro-MMP activity may be declining viability of the mesenchymal cells, cytotrophoblasts and syncytiotrophoblast responsible for MMP production in the explants at various times during culture (section 3.7.1), resulting in a decrease in protein synthesis, and a breakdown in the cytokine signaling network which is likely to be operating between these cell types. Therefore, the vast majority of previously synthesized pro-MMP may have been converted to active forms during the explant culture period but new pro-MMP may not have been synthesized.

**A.4 Conclusion**

Both MMP-2 and MMP-9 are expressed by first trimester villous explants cultured in both 8% and 1.5% oxygen conditions. However, differences in MMP production in different oxygen concentrations, or in explants that had or had not produced extravillous trophoblast outgrowth were not able to be accurately quantified as this work has shown
that there are several major problems with the use of gelatin zymography as a quantitative method for MMP activity. Firstly, the total variation of identical samples is a significant percentage of the range of expected MMP activity. This means that gelatin zymography was not sensitive enough to distinguish differences in MMP activity at the level required for this work. Secondly, because of the large amount of inter-zymogram variation, in order to obtain accurate results a complete standard curve would need to be run on each zymogram, which would take up a significant proportion of each zymogram. Thirdly, even if a standard curve was run on each zymogram, preliminary data indicates that these standard curves are not reproducible. These factors resulted in the decision that gelatin zymography was not practical or sensitive enough for obtaining accurate results to determine the difference in MMP activity between individual explants.

Finally, this work has shown that gelatin zymography may be able to be used as a quantitative method of determining MMP activity in cases where large changes in activity are present and a high level of sensitivity is not required. However, these data call into question the methodologies employed by a number of researchers using gelatin zymography as a quantitative method because of the high level of inter-zymogram variation. A number of studies do not specifically describe how they have analyzed their zymograms, or controlled for the possibility of inter-gel variation (Canning et. al., 2001; Graham & McCrae, 1996; Niu et. al., 2000). Therefore, caution should be taken when interpreting supposedly quantitative gelatin zymography results.
Appendix B:

Development of Controlled Oxygen Environment Culture System
B.1 Introduction and rationale

Low oxygen conditions were required in vitro in order to examine the response of villous explants to the low oxygen conditions present until approximately 10 weeks of gestation in vivo (Chapter 5) (Jauniaux et al., 2003b; Jauniaux et al., 1992). As trophoblast oxygen detection mechanisms are sensitive, the accuracy of the oxygen concentrations used to examine the effects of low oxygen on EVT outgrowth were important for overall experimental accuracy in the explant system. The target oxygen concentration to mimic the period before maternal blood enters the IVS was between 1-2% oxygen, whereas 8% oxygen was used to mimic arterial blood oxygen concentrations that the placenta would be exposed to after this time (Howard et al., 1961; Rodesch et al., 1992). Other researchers have previously shown that atmospheric oxygen concentrations correlate well with dissolved oxygen concentrations in media after 24 hours at both 18% and 2% oxygen (Newby et al., 2005). Initial attempts to use a larger airtight chamber previously reported to be used for hypoxic culture were unsuccessful, and consequently a system of culture in blood bags was developed.

B.2 Measurement of oxygen concentrations

A fyrite gas cylinder (1-7%) (Bacharach, USA) was used according to the manufacturers’ instructions to determine the oxygen concentration within the chamber/bag being tested. Certified beta-standard gas mixes of 2.5% O₂, 1% O₂ or 8% O₂ each with 5% CO₂ in N₂ (BOC, Auckland) were used to trial equipment and establish cultures. The accuracy of the fyrite equipment was ensured by first measuring oxygen samples directly from the certified gas mixtures in triplicate. As this setup contained lengths of tubing which could be a potential source of oxygen contamination from the ambient air, all tubing was flushed with the lowest concentration of oxygen available to ensure minimal contamination and accurate measurement. Using this method the fyrite cylinder accurately indicated the oxygen concentration in all gas mixtures (1% O₂ mix ±0.03% O₂, 2.5% O₂ mix ±0.03% O₂, 8% O₂ mix ± 0.01% O₂).
B.3 Determination of oxygen concentrations in a modular incubation chamber

Previous research in the IVF field has utilized a modular incubation chamber (MP Biomedicals, USA) for low oxygen cultures. Therefore, this product was obtained to culture explants in low oxygen conditions (Figure B.1). In order to ensure that flushing this chamber with low oxygen gas mix would result in the equilibration of the atmosphere with the flushed gas, the chamber was flushed with the 2.5% O₂ containing gas mixture for several different time periods and the oxygen content of the chamber was measured by fyrite cylinder analysis (Table B.1). However, in this initial trial the chamber failed to reach the low oxygen concentrations required (2% oxygen) (Table B.1).

![Figure B.1 – Photograph of the modular incubation chamber (MP Biomedicals, USA) trialled for use as a oxygen controlled culture system. The chamber is shown with a dummy plate of media inside.](image)

Therefore, in order to assist in decreasing the oxygen content in the chamber more efficiently, the source gas mixture was changed to 1% oxygen and the chamber was adapted with tubing so that the incoming gas was released on the opposite side from the outlet port. In a further attempt to fill the chamber with inlet gasses the inlet was
adapted with a 6 outlet manifold. However, these modifications failed to lower the oxygen concentration in the chamber to suitable experimental levels (Table B.1). Humidifying the airflow by passing it through de-ionized H₂O did not assist in further lowering the oxygen content in the chamber (Table B.1).

<table>
<thead>
<tr>
<th>Incubation Chamber Setup</th>
<th>Input Oxygen Concentration (%)</th>
<th>Input Gas Pressure (psi)</th>
<th>Length of Flush (min)</th>
<th>Final Oxygen Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>2.5</td>
<td>2.5</td>
<td>2</td>
<td>&gt;10⁰</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.5</td>
<td>5</td>
<td>&gt;10⁰</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2.5</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>4</td>
<td>16</td>
<td>8 (±0.9)</td>
</tr>
<tr>
<td>Adapted</td>
<td>1</td>
<td>2.5</td>
<td>10</td>
<td>8 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.5</td>
<td>15</td>
<td>5.6 (±0.7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.5</td>
<td>20</td>
<td>4.5 (±0.2)</td>
</tr>
<tr>
<td>Humidified</td>
<td>1</td>
<td>4</td>
<td>10</td>
<td>7.2 (±0.3)</td>
</tr>
</tbody>
</table>

Table B.1 – Evaluation of oxygen concentrations obtained in a modular incubation chamber. Flushing the chamber as purchased (standard setup) did not result in oxygen concentrations equal to the input gas concentration. When the chamber was modified so that the inlet port was on the other side of the chamber from the outlet port and gas mixing of the input gas was increased (adapted setup) and the concentration of oxygen in the input gas mixture was reduced, however not to levels low enough for experimental explant culture. Humidifying the airflow did not affect the final oxygen concentration. Final oxygen concentration values in the table represent the average of duplicate experiments. No standard error is provided for measurements in which all values were off the scale of the yfrite cylinder (*).

Finally, the flow of gas in the chamber was visualised using an air current tube (Draeger, Luebeck, Germany) which generated smoke so that the airflow in the chamber could be visualized. This showed that the input gas was not mixing throughout the chamber. On the basis of the above experiments, use of the modular incubation chamber was abandoned and a new system of culturing explants under low oxygen conditions was developed.
B.4 Establishment and accuracy of a low oxygen culture system in blood bags

In order to be able to culture explants in a smaller volume of gas, allowing gas levels to be established more efficiently and accurately, 1L capacity blood bags were adapted for the culture of 96 well plates. The end of the bag was cut open to allow insertion of a culture plate and resealed with a heat sealer, bags were inflated, then inlet and outlet ports were clamped shut. When empty bags were inflated to full capacity with N₂ and left for 24 hours bags showed no sign of deflation, establishing that the bags were effectively airtight. However, the oxygen concentration within the bag increased from 0% oxygen in freshly flushed bags to 2% oxygen after 24 hours. As there was no discernable leakage, and the bags were under positive pressure, it is unclear how this increase in oxygen concentration occurred.

Figure B.2 – Photograph demonstrating the blood bag controlled oxygen culture system developed. A dummy plate with media is shown sealed inside an inflated 1L blood bag. Inlet and outlet ports are sealed shut and the end of the bag cut open to insert the bag (right hand side of the image) has been heat sealed shut.

When dummy media-filled 96 well plates were sealed in blood bags and inflated with N₂ the oxygen contained in the media resulted in an increased concentration of oxygen present in the blood bag after inflation such that when gas levels were evaluated by fyrite cylinder analysis 30 minutes after flushing, the oxygen concentration in the bags had a
mean of 6.2% (Table B.2). In order to allow the oxygen from the media to diffuse into the surrounding N₂, bags were flushed for 1 minute at 30 minute intervals. This resulted in a steady decline in the oxygen content in the bags (Table B.2). This methodology was further refined by increasing the size of the outlet holes in the bags, and decreasing the interval between flushing, resulting in the desired oxygen concentration of the model input gas (0% oxygen) (Table B.2). This methodology was then trialled using the gas mixtures required to obtain experimental low oxygen concentrations (1% O₂, 5% CO₂ in N₂). Three consecutive 1 minute cycles with 15 minute incubations at 37°C resulted in an average oxygen concentration of 1.2%. However, when incubated at 37°C for 24 hours the oxygen concentration in the bags increased to 2% oxygen. In order to remedy this situation and maintain oxygen concentrations between 1-2% oxygen, the blood bags were flushed daily for 1 minute throughout each experiment. Therefore, the low oxygen conditions employed in this thesis will be defined as 1.5% oxygen, representing the average oxygen value over the 24 hour period between flushings.
<table>
<thead>
<tr>
<th>Experimental Design</th>
<th>Input Gas and Concentration</th>
<th>Time (hrs)</th>
<th>Oxygen Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood bags were emptied and refilled every 30 minutes</td>
<td>100% N₂</td>
<td>0.5</td>
<td>6.2 (±0.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>6.2 (±0.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2</td>
<td>1.8 (±0.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.2 (±0.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>0 (±0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.4 (±0.2)</td>
</tr>
<tr>
<td>Blood bags were filled then emptied and refilled every 20 minutes.</td>
<td>100% N₂</td>
<td>1</td>
<td>2.1 (±1.1)</td>
</tr>
<tr>
<td>Blood bags were flushed every 30 minutes for 1 minute</td>
<td>100% N₂</td>
<td>2</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>Blood bags were flushed every 20 minutes for 1 minute</td>
<td>100% N₂</td>
<td>1</td>
<td>0.4 (±0.4)</td>
</tr>
<tr>
<td>Blood bags modified to increase the size of the output hole were flushed every 15 minutes for 1 minute.</td>
<td>100% N₂</td>
<td>0.75</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>Blood bags modified to increase the size of the output hole as above were flushed every 15 minutes for 1 minute.</td>
<td>1% O₂, 5% CO₂ in N₂</td>
<td>0.75</td>
<td>1.2 (±0.1)</td>
</tr>
</tbody>
</table>

Table B.2 – Table showing the results of the experiments used to optimise the low oxygen protocol developed for explant culture. 96 well plates with 80 wells each containing 150µL of DMEM/F12 were sealed in the blood bags. Then the bags were either emptied and refilled, or flushed at a pressure sufficient to just inflate the bag to capacity with the outlet port open. The
time shown is the time at which the oxygen content of the bags were measured from the start of the flushing protocol. This allowed the development of the most efficient method of reaching the input gas concentration in the blood bags. All values shown are the averages of duplicate or triplicate experiments.

When a mixture of 8% O₂, 5% CO₂ in N₂ was flushed as described above, the blood bags contained the target oxygen concentration of 8% oxygen. When the blood bags were incubated for 24 hours at 37°C the oxygen concentration in the bags increased by 0.4% (∓). Therefore, in order to maintain oxygen concentrations at 8%, and to ensure both 1.5% and 8% cultures were exposed to the same protocols, the bags containing 8% oxygen were also flushed with 8% O₂, 5% CO₂ in N₂ daily for 1 minute. As a final quality control measure, the levels of oxygen in 30 bags that had been used for explant cultures and had been established and flushed daily as described were measured by fyrite at the end of a 5 day culture period (see Chapter 5). All blood bags had the expected final oxygen concentration of 2% (∓0.03) or 8% (∓0.02).

**B.5 Conclusion**

It is important to establish an accurate culture system to study trophoblast differentiation from villous explants in low oxygen conditions. Equipment used by others was found not to establish or maintain accurate low oxygen conditions. To overcome this problem, a novel method of culturing explants in low oxygen conditions was developed using blood bags. This methodology proved to be and accurate, cost effective and reliable method of culture in controlled oxygen conditions.
Appendix C:

Publications Arising from this Thesis
Cytotrophoblast differentiation in the first trimester of pregnancy: evidence for separate progenitors of extravillous trophoblasts and syncytiotrophoblast

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Abstract

It is commonly accepted that a single pool of villous cytotrophoblasts are precursors of both syncytiotrophoblast and extravillous trophoblasts during the first trimester. Here we present evidence that these two trophoblast subpopulations arise from separate progenitors that have different survival characteristics when studied in villous explant cultures. Dual staining with chloromethylfluorescein diacetate and ethidium bromide revealed degeneration of the syncytiotrophoblast by non-apoptotic mechanisms within 4 h of culture. The syncytiotrophoblast had regenerated within 48 h but at this point the vast majority of the cytotrophoblast and cells of the mesenchymal core were dead. Despite this extensive cytotrophoblast death, explants are able to produce extravillous trophoblast outgrowth for up to 3 weeks in culture. We believe that the villous cytotrophoblasts in the tips of anchoring villi are resistant to the factors that cause the death of the majority of villous cytotrophoblasts in culture. We speculate that as early as 8 weeks of gestation there are two separate villous cytotrophoblast populations, one committed to differentiate into syncytiotrophoblast and the second committed to the extravillous differentiation pathway.

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Introduction

The human placenta is a fetal organ that is essential for the development of the embryo and the success of pregnancy. The placenta acts as an interface between the mother and developing fetus, playing important roles in implantation and nutrient and gas transport, along with immune, endocrine and metabolic functions (Cross et al. 1994). In the first trimester of human pregnancy the placenta has a villous structure. Placental villi contain fetal blood vessels and occasional macrophages (Holbauer cells) in a core of mesenchymal connective tissue, surrounded by a layer of mononuclear cytotrophoblast stem cells that is overlain by a continuous layer of multinucleated syncytiotrophoblast.

Villus cytotrophoblast stem cells differentiate along one of two possible pathways, either fusing to form the overlying syncytiotrophoblast or, in anchoring villi (those villi that physically attach the placenta to the uterus), invasive cytotrophoblasts break through the syncytiotrophoblast to form extravillous trophoblast columns (Aplin 1991). Extravillous trophoblast columns invade into the maternal decidua, physically connecting the placenta to the decidua (Piijnenborg et al. 1980, Kam et al. 1999). As the extravillous trophoblast columns move away from the anchoring villi they also spread laterally around the placenta and invade the maternal spiral arteries (Brosens et al. 1967, Kam et al. 1999). Invasion of the spiral arteries by extravillous trophoblasts leads to transformation of these vessels into large bore conduits which are necessary to allow the increased maternal blood flow that is required by the placenta/fetus as pregnancy progresses. Transformation of the spiral arteries by extravillous trophoblasts is called the ‘physiological changes of pregnancy’ and inadequate physiological changes of pregnancy are found in pregnancies complicated by pre-eclampsia and intra-uterine growth restriction (Brosens et al. 1967, Khong et al. 1986). Extravillous trophoblast invasion is tightly regulated both temporally and spatially, and is essential for the success of pregnancy (Cross et al. 1994, Morrish et al. 2001). The factors which drive the differentiation of villous cytotrophoblasts into either syncytiotrophoblast or extravillous trophoblast are not known. A number of interacting factors have been implicated in the migration of extravillous trophoblasts away from the placenta; however, it remains unclear which factors are essential for this process.

In this study, we have used explant cultures of first trimester human placenta to examine the behaviour of
various trophoblast populations. Our research has led us to question the commonly held concept that a single pool of villous cytotrophoblasts are precursors of both syncytiotrophoblast and extravillous trophoblast. Rather, our results suggest that even as early as 8 weeks of gestation two separate pools of villous cytotrophoblast exist which appear to be committed to differentiation into either syncytiotrophoblast or extravillous trophoblast.

**Materials and Methods**

This study was approved by the regional Ethics Committee and all tissue samples were obtained with informed consent.

**Explant culture**

First trimester placenta were obtained following elective surgical termination of pregnancy (TOP) and washed gently in phosphate-buffered saline (PBS; pH 7.4). The gestational age and fetal viability of all pregnancies prior to TOP were confirmed by ultrasound assessment. Matri
gel (Becton Dickinl, Sydney, Australia) was thawed slowly at 4°C and diluted to 1:10 in Dulbecco's modified Eagles' medium salts/FS12 at 4°C (Life Technologies, Auckland, New Zealand). Wells of sterile 96-well culture plates (Falcon, Sydney, Australia) were coated with 50μg/well 10% Matrigel and incubated at 37°C for 25 min. Excess Matrigel was removed, leaving a thin coat on each well. Villous tips were gently teased from the placenta, separated into pieces of approximately 8mg wet weight, and placed in the centre of each well. The villous explants were incubated at 37°C for 5 min, and then 150μl/well complete trophoblast medium (DME/F12 containing 10% fetal bovine serum, 5 mg/ml epidermal growth factor, 5 μg/ml insulin, 10 μg/ml transferrin, 100 μg/ml streptomycin, 20 mM sodium selenite, 400 IU human chorionic gonadotrophin and 100 IU penicillin) was added. The plate was then centrifuged at 210g for 1 min in order to facilitate adhesion. The explants were cultured at 37°C in a humidified ambient oxygen atmosphere with 5% CO2. Two-dimensional outgrowth of trophoblasts from the explants across the thin layer of Matrigel was observed directly by phase contrast microscopy using a Nikon E1 WD 0.3 phase contrast microscope with a Ph1 10 DL/0.25 Numerical Aperture (N.A.) lens (Nikon, Tokyo Japan).

**Assessment of cellular viability by confocal microscopy**

In total, 164 explants from 12 placentae of 8 to 12 weeks of gestation were incubated with 5μM 5-chloromethylflourescin diacetate (CMFDA) (Molecular Probes, Eugene OR, USA) in complete trophoblast medium at 37°C for 1.5h. The medium was then replaced every 10 min for 30 min with complete trophoblast medium only. Explants were then incubated with 2.5 μg/ml ethidium bromide (EtBr) (Sigma) in PBS, pH 7.4 at room temperature for 1 min, and washed four times in PBS, pH 7.4. At each time-point, one explant was treated with Virlkon (Biolab, Auckland, New Zealand) for 10 min before staining to serve as a control of cell death. Jeg-3 choriocarcinoma cells were used as a control to indicate cell viability. Explants were visualized in PBS at room temperature on a Leica TC SP2 confocal microscope (Leica, Oberkochen, Germany) using Leica HC PL APO 20 ×/0.7 N.A. Immersion Correlation Confocal Scanning and HCX PL APO 40 ×/1.25 N.A. Oil/Ph3 CS lenses (Leica) and photomicrographs recorded using Leica confocal software version 2.5.1104. Figures were compiled using Adobe Photoshop v5.0 (Adobe Systems, San Jose, CA, USA).

**Assessment of apoptosis by DNA laddering assay**

In parallel to assessment of cellular viability by confocal microscopy, at 0, 48 and 96 h of culture 25 explants from each of the cultures established for confocal microscopy were homogenized with 500 μl lysis buffer (4M guanidium thiocyanate, 1% N-lauryl sarcosyl and 10 mM dithiothreitol) and incubated in a 37°C water bath for 30 min to settle. DNA was extracted from the homogenate by the method of Daniel et al. (1999). DNA was stored at 4°C, or at −20°C for prolonged storage. Twenty-five microlitres of DNA extract and 3 μl xylene cyanol dye (0.25% xylene cyanol and 30% glycerol in H2O) were loaded onto a 2% (w/v) agarose gel and run at 100 V. A positive control of DNA extracted by the same method from U937 cells incubated with 5 μM camptothecin for 5 h at 37°C to induce apoptosis was run run every gel. Gels were stained in 0.5 μg/ml EtBr (Sigma) in TAE buffer (40 mM Tris–HCl, 20 mM glacial acetic acid and 1 mM EDTA in H2O) for 20 min, rinsed in water twice for 10 min and visualized on a u.v. lightbox.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Fresh tissue from three 8-week placenta was divided into explants which were incubated with 200 μl/well complete trophoblast medium in a 96-well plate. At hourly intervals up to 5 h from the establishment of culture, 20 μl 5 mg/ml MTT (Merck, Darmstadt, Germany) was added to three wells for 1 h. Liquid nitrogen was then used to freeze MTT-treated explants in cryo-embedding medium (Biotek Scientific Supplies, Auckland, New Zealand).

The frozen tissue blocks were cut into 5 μm sections using a cryostat (Leica CM1900) and collected on glass slides (BioLab Scientific, Auckland, New Zealand). Slides were fixed in 4% (w/v) paraformaldehyde in PBS for 3 min and air dried for 1 h. Slides were mounted with Aquamount (Biotek Scientific Supplies) and visualized at room temperature using Nikon plan achromat lenses of 10 ×/0.25 N.A. and 20 ×/0.4 N.A. on a Nikon E400 light microscope. Images were captured using a
Nikon Coolpix 990 digital camera and downloaded into Microsoft PhotoEditor v3.0.2.3 (Microsoft, Seattle, WA, USA).

**Immunohistochemistry**

First trimester placental tissue was divided into pieces of approximately 4 mm³ at the time of collection, or explants were removed from cultures at 4, 8, 24, 48 or 96 h. Tissue was frozen and sectioned as described above. Slides were dipped in de-ionized H₂O for 3 s, dried for 1 h, then fixed by immersion in cold acetone for 10 min, air dried and stored at −20°C.

Non-specific binding was blocked by the addition of 100 µl 10% normal goat serum in PBS-Tween (Life Technologies) for 10 min at room temperature. The slides were then washed three times with PBS, pH 7.4, containing 0.05% Tween 20 (PBS-Tween). Tissue sections approximately 25 µm apart were covered with 100 µl of either 1:20 M30 cytoeath antibody (Roche, Penzburg Germany) or 1:200 activated Caspase 3 antibody (Sigma) diluted in 10% normal goat serum in PBS-Tween for 1 h at room temperature. The slides were then washed three times with PBS-Tween and endogenous peroxidase activity was quenched by the addition of 50 µl 3% H₂O₂ in methanol for 5 min. The slides were then washed three times with PBS-Tween. A Zymed Histostain-Plus kit (Zymed, San Francisco, CA, USA) containing biotinylated secondary antibody and enzyme conjugate was used according to the manufacturer’s instructions. Amino ethyl carbamyl stain (Medbio, Christchurch, New Zealand) was used according to the manufacturer’s instructions. Slides were then washed with de-ionized water, immersed in haematoxylin nuclear stain (Surgipath; Australian Laboratory Services, Auckland, New Zealand) for 1 min, then washed with tap water and coverslips were mounted with Aquamount. Slides were observed at room temperature by light microscopy (Nikon Eclipse E400) as before and images captured using a Nikon Coolpix 990 digital camera. Figures were compiled using Adobe Photoshop v5.0.

**Explant passage cultures**

Three hundred and eighty-four explants from four placenta (two each of 9 and 11 weeks of gestation) were cultured using the methods described above (primary passage culture). On the seventh day of culture, villous explants were transplanted into a secondary passage culture by placing them into a well of a fresh Matrigel-coated plate. One hundred and fifty microlitres of fresh complete trophoblast media were added and the culture was continued for 7 days. Villous explants from the secondary passage culture were transplanted by the same method to a tertiary passage culture and the culture continued for 7 days. The outgrowth of extravillous trophoblasts from the explants in each passage was observed directly by phase contrast micrography using a Nikon EL WD 0.3 phase contrast microscope with a Nikon Phl 10 DL/0.25 N.A. objective lens, and recorded on days 1, 2, 3, 4 and 7 of each culture using a Nikon Coolpix 990 digital camera. Prolonged cell viability in a placental explant culture of 8.2 weeks of gestation was assessed by staining with 5 µM CMFDA and 2.5 µg/ml EBBr at the end of the second passage, as described above. Tips producing extravillous trophoblast outgrowth were imaged using an inverted modified Zeiss LSM 410 confocal microscope (Zeiss, Oberkochem, Germany).

**Results**

**Viability of cell populations in first trimester villous explants**

In order to investigate the viability of trophoblasts in villous explants in culture, explants from nine placentae were cultured for up to 96 h. Explants were stained with CMFDA, a cell-permeable dye which is metabolized in viable cells to a cell-impermeant fluorescent green dye, and EBBr which is only able to enter cells when membrane integrity has been compromised indicating cell death, and visualized by confocal microscopy at 4, 48 and 96 h. After 4 h in culture, the earliest time-point measurable by this method, the viability of the syncytiotrophoblast layer was severely compromised as revealed by uptake of EBBr and failure to metabolize CMFDA (Fig. 1a). After 24 h, fragments of dead syncytiotrophoblast were observed to be shedding from the explant (Fig. 1b). As the culture continued to 96 h significant areas of viable syncytiotrophoblast were evident, but the villous cytotrophoblasts underlying the syncytiotrophoblast as well as the cells of the mesenchymal core were no longer viable (Fig. 1c and d).

The MTT assay was used to assess cellular viability between 0 and 5 h in culture. After 1 h in culture all cell types in the villi were viable (Fig. 2a). The MTT staining in the villous cytotrophoblast was often more intense than that in the syncytiotrophoblast or cells of the mesenchymal core (Fig. 2a). From 4 h of culture, approximately half of the syncytiotrophoblast was non-viable, whereas the cytotrophoblast and cells of the mesenchymal core remained viable (Fig. 2b).

**Apoptosis is not the dominant cause of cell death in cultured villous explants**

In order to examine whether the loss of cellular viability in cultured villous explants was due to apoptotic death, DNA was extracted from pooled explanted villous tissue and examined for DNA laddering. DNA laddering was virtually absent from freshly harvested villous tissue, with a faint ladder present in DNA from only one of nine placentae, but DNA laddering became more frequent as the length of culture continued and was apparent in explants from six of nine placentae from 48 h of culture (data not shown).
In order to determine which cells in the villous explants were apoptotic, the expression of activated caspase-3 and a cytokeratin neoeptope (M30), created by cleavage of cytokeratin 18 by activated caspases, was examined by immunohistochemistry (Fig. 3). Freshly harvested villous tissue did not stain with the cytokeratin neoeptope M30 antibody and only rare villous cells contained activated caspase-3. The activated caspase-3-positive cells were primarily confined to the mesenchymal core of the villi, suggesting that apoptosis was not occurring in the syncytiotrophoblast of this fresh tissue (Fig. 3). Only small stretches of syncytiotrophoblast and occasional cytotrophoblasts stained for activated caspase-3 from 4 h in culture, or for the M30 cytokeratin neoeptope from 48 h of culture (Fig. 3). Increased expression of activated caspase-3 in the cells of the mesenchymal core was seen with extended culture, although the levels of expression in these cells varied greatly between individual explants. Although there was sporadic apoptotic death of all cell types in the explants, by and large trophoblast death appeared to be primarily non-apoptotic.

**Figure 1** Optical sections of explants from a 12.2-week placenta stained with 5 μM CMFDA and 2.5 μM EBBr after (a) 4, (b) 24, (c) 48 and (d) 96 h of culture and visualized by confocal microscopy. (a) After 4 h the syncytiotrophoblast is not viable, as shown by the uptake of EBBr; however, the cytotrophoblasts and cells of the mesenchymal core metabolise CMFDA and exclude EBBr. (b) The non-viable syncytiotrophoblast layer is shed in syncytial knots as observed after 24 h of culture. (c) However, after 48 h of culture the syncytiotrophoblast has regenereated and is able to metabolise CMFDA, whereas the cytotrophoblast and cells of the mesenchymal core are no longer viable. (d) The pattern of staining seen after 48 h of culture is repeated after 96 h of culture.

**Figure 2** (a) Photomicrograph showing MTT staining of villous tissue from an explant of 8.4-weeks of gestation 1 h after collection. MTT staining (purple) is present in the syncytiotrophoblast, cytotrophoblast and cells of the mesenchymal core. MTT staining in the cytotrophoblast layer (short arrow) appears darker than that of the syncytiotrophoblast (long arrow). (b) Photomicrograph showing MTT staining of villous tissue from an explant of 8.4-weeks of gestation 4 h after collection. The syncytiotrophoblast (long arrow) is no longer viable, as shown by an absence of MTT metabolism. The cytotrophoblast (short arrow) and mesenchymal cells remain viable.
Trophoblast outgrowth continues despite the death of most villous cytotothrophoblasts

Of the explants cultured for 96h, 19.1% produced areas of extravillous trophoblast outgrowth. A comparison of those explants that produced extravillous trophoblast outgrowth with those that did not produce outgrowth showed no difference in the viability of either the syncytiotrophoblast or villous mesenchymal core. Extravillous trophoblast outgrowth became macroscopically obvious by 96h in culture despite the death of most of the villous cytotothrophoblasts and cells of the villous mesenchymal core and continued to expand until it was limited by the destruction of the thin layer of Matrigel used in our culture system. At this point, the only villous cytotrophoblast population that remained viable were the pockets of multilayered cytotothrophoblast that were located in villous tips directly behind the extravillous trophoblast columns (Fig. 4).

In order to examine whether extravillous trophoblast outgrowth would continue for extended periods, villous explants from four placentae were cultured for 7 days (primary culture) then transferred to fresh Matrigel-coated culture plates. The culture was continued for a further 7 days (secondary culture) and the explants were again transferred to fresh plates for an additional 7 days of culture (tertiary culture). In these experiments, 32.6, 8.0 and 3.2% of the explants produced extravillous trophoblast outgrowth in the primary, secondary and tertiary cultures respectively. Interestingly, 25% of explants that produced trophoblast outgrowth in the second passage did not produce extravillous trophoblast outgrowth in the first passage. At the end of the second passage culture, CMFDA and EBr staining confirmed that the majority of cells in the villus were not viable (Fig. 5). However, the cytotothrophoblasts in the villous tips from which outgrowth was produced, and the outgrowth itself, were viable (Fig. 5).
**Discussion**

In order to study extravillous trophoblast differentiation, our laboratory has developed a first trimester villous explant model in which pieces of villous tissue are cultured on a very thin coating of Matrigel (an extracellular matrix substance). Unlike other models employing Matrigel, the thin coat of Matrigel means that extravillous trophoblast outgrowth in this model is two-dimensional and can therefore be readily quantified by phase-contrast microscopy and digital image analysis. Outgrowth produced from first trimester villous explants in this model was morphologically distinct and stained with antibodies against cytokeratin 7, but not vimentin, confirming that it consists only of extravillous trophoblasts.

When establishing this model we were concerned by reports that the syncytiotrophoblast rapidly degenerates in explant cultures (Watson et al. 1995, 1998, Palmer et al. 1997, Siman et al. 2001). Palmer et al. (1997) studied cultured first trimester chorionic villi by electron microscopy, and demonstrated degeneration of the syncytiotrophoblast by 24 h of culture. A new syncytiotrophoblast layer was then formed from the viable underlying cytotrophoblasts by 48 h in culture, and was maintained until at least 120 h of culture (Palmer et al. 1997). We have confirmed this finding using CMFDA, an indicator of cell viability, and EtBr, an indicator of cell death. The exclusion of EtBr and metabolism of CMFDA provide two independent and objective measures by which viability can be assessed simultaneously in individual cells. Using these markers, we have shown that the vast majority of the syncytiotrophoblast is non-viable after 4 h in culture and that large areas of the dead syncytiotublast are shed by 24 h in culture. It seems likely that, despite very gentle treatment of the tissues, the initial massive syncytiotrophoblast death was triggered in the early stages of culture or during the preparation of the tissue. The syncytiotrophoblast also rapidly lost the ability to metabolize MTT after the placenta were harvested, but in contrast to the uptake of EtBr and lack of CMFDA metabolism over the entire syncytiotrophoblast after 4 h in culture, significant areas of syncytiotrophoblast retained the ability to metabolize MTT at this time-point. However, care must be taken in using MTT as an indicator of cellular viability as the mechanism of cellular MTT reduction is not completely understood (Liu et al. 1997). MTT is generally accepted to be reduced by the mitochondrial electron transport chain, based on a study of cell homogenates by Slater et al. (1963). However, in intact cells, MTT is also able to be reduced by NADH- and NADPH-dependent mechanisms in intracellular vesicles, and is therefore able to be affected by factors such as oxidative stress (Liu et al. 1997). Exposure of early first trimester villous tissue, which exists in a hypoxic environment in vivo and does not contain protective antioxidant enzymes, to atmospheric oxygen concentrations would increase cellular levels of reactive oxygen species (Watson et al. 1997). High levels of reactive oxygen species have been associated with an over-reporting of cellular viability using MTT (Collier & Priftos 2003).

After 24 h in culture we observed the formation of syncytiotubular ‘knots’ that are involved in the process of syncytiotrophoblast shedding from the villus. The extrusion of terminally differentiated syncytiotubular through the formation of syncytiotubular ‘knots’ is essential for the renewal of the syncytiotrophoblast and the growth of the placenta (Huppertz et al. 1998, 1999, Hempstock et al. 2003). In our cultures, the syncytiotrophoblast layer was partially regenerated as the culture progressed beyond 24 h, whereas the underlying cytotrophoblast layer was largely non-viable. We propose that the regenerated syncytiotrophoblast was formed from the cytotrophoblasts that were viable during the first 1–2 days of culture. The syncytiotrophoblast regeneration did not completely deplete the population of underlying cytotrophoblasts, as a non-viable cytotrophoblast layer remained present at later time-points, although this layer was discontinuous in places. It is not clear why these cytotrophoblasts and the cells of the mesenchymal core died with prolonged culture, but possibly a lack of...
specific growth factors from the damaged syncytiotrophoblast or the loss of the fetal circulation contributed to the death of these cells. It is also important to note that the structure and cellular constituents of the mesenchymal core change as the villi mature and become more cellular with advancing gestation. Thus, the anchoring villi that this study concentrated on may not be entirely representative of large villi such as stem villi. Since massive cell death had occurred in these cultures the question may be asked as to why the explants remain intact. In the sterile culture system we describe there is no bacterial breakdown of the tissues. In vivo, cells of the immune system, particularly macrophages, would be responsible for removing dead cells, but in this in vitro system there are only low levels of macrophages contained in the villous core and consequently the dead but sterile tissue might be expected to remain intact for prolonged periods of time as we have observed.

The rare staining of the syncytiotrophoblast with antibodies to the cytokeratin neoeptope M30 and activated caspase-3 as well as the absence of DNA laddering that we observed suggest necrosis rather than apoptotic death of the syncytiotrophoblast, particularly during the first 24 h of culture. Apoptotic cell death does occur during the later stages of explant culture, but staining for activated caspase-3 and the cytokeratin neoeptope M30 indicated that this arises from low level sporadic apoptotic death of all villous cell types and that much of the cell death that occurs during the later stages of culture is non-apoptotic. These results are in contrast to previous findings of high levels of trophoblast apoptosis within 24 h in term villous explants also detected using the cytokeratin neoeptope M30 antibody (Di Santo et al. 2003). However, it is possible that such differences in the level of observed apoptosis represent intrinsic differences between placentae in the first trimester and at term. It may also be possible that cell death pathways not involving the caspase pathway have been activated.

The continuation of trophoblast outgrowth from villous explants for up to 3 weeks in culture, despite the widespread death of the trophoblasts underlying the syncytiotrophoblast, indicated that the ability of villi to produce extravillous trophoblast outgrowth and support the expansion of extravillous trophoblast columns cannot be dependent on either the cells of the mesenchymal core or the majority of the villous trophoblasts, which are largely non-viable within the first week of culture. In contrast to the majority of villi, those villi from which extravillous trophoblast columns originate contain multiple layers of villous trophoblasts at their tips (Vivacav et al. 1995). We have found that these multilayered villous trophoblasts at the origins of trophoblast columns remain viable during prolonged culture. It is widely accepted that villous trophoblasts from term placentae are committed to differentiate into syncytiotrophoblast (Morrish et al. 1997). Based on the evidence from our study we propose that as early as 8 weeks of gestation there are two distinct populations of villous cytotrophoblasts. (1) the majority of villous cytotrophoblasts form a monolayer directly beneath the syncytiotrophoblast. These cells do not survive well in the culture conditions we employed and we propose that they are committed to fusion into syncytiotrophoblasts. We will refer to these as monolayer villous cytotrophoblasts. (2) Villous c.
(Bischof et al. 2000, Lacey et al. 2002). However, despite the death of the vast majority of cells in the villi within 4 days of culture, extravillous trophoblast outgrowth continued for up to 3 weeks in our model. This suggested that extravillous trophoblast progenitors are not solely dependent upon paracrine signals to drive their differentiation or invasive capacity. However, it is likely that paracrine factors would enhance trophoblast outgrowth. Alternatively, sufficient growth factors may be derived from the Matrigel, which, although used as a very thin coat of diluted (10%) Matrigel on the culture wells in this model, remains a potential source of cytokines and growth factors. The behaviour of cells in culture is dependent on the conditions of culture employed. Many workers use deep layers of concentrated Matrigel which give three-dimensional cultures. In these other models it is possible that relatively large quantities of factors that promote cell survival or other cellular behaviour are supplied by the Matrigel and thus our results may not be directly compatible with other models which use much larger amounts of Matrigel.

Other groups have previously reported phenotypic markers that distinguish the two trophoblast populations that we describe. Tenascin, an extracellular matrix glycoprotein, is present in anchoring villi immediately adjacent to sites of cytotrophoblast column initiation, but is not detected on monolayer villous cytotrophoblasts (Castellucci et al. 1992, Damsky et al. 1992). The receptor for tenascin, αvβ6 integrin, is also expressed only by villous cytotrophoblasts at sites of extravillous trophoblast column initiation, and not in monolayer cytotrophoblasts or other villous cell types (Zhou et al. 1997). Furthermore, the binding of tenascin by αvβ6 integrin stimulates cellular proliferation (Yokosaki et al. 1996). Proliferation of extravillous trophoblast progenitors would be required to drive expansion of the extravillous trophoblast columns as extravillous trophoblasts in columns do not proliferate (Vivovac et al. 1995, Korhonen & Vittanen 1997). In contrast to extravillous trophoblast progenitors, only a small proportion of monolayer villous cytotrophoblasts stain with the proliferation marker Ki67, demonstrating another key difference between monolayer villous cytotrophoblasts and extravillous trophoblast progenitors (Vivovac et al. 1995).

Our findings that villous cytotrophoblasts from first trimester placenta do not represent bipotent progenitors could explain why it is difficult to obtain large numbers of trophoblasts that either differentiate into an invasive extravillous phenotype or proliferate following enzymatic digestion of first trimester placenta, as the vast majority of villous cytotrophoblasts are contained in the villose monolayer and we believe they are committed to the syncytiotrophoblast differentiation pathway.

The rapid degeneration of the syncytiotrophoblast in this model raises some concerns for the use of explant models in the study of syncytiotrophoblast function. In such studies, researchers should be aware of the rapid degeneration of the syncytiotrophoblast and design experiments taking this into account. However, as the observed cell death does not affect the ability of explants to produce extravillous trophoblast outgrowth, and viable trophoblast outgrowth is able to be produced for up to 3 weeks in culture, the explant model remains a good method for the study of trophoblast invasion.

In summary, in this study using a villous explant model, we have confirmed rapid death of the syncytiotrophoblast by a non-apoptotic mechanism. However, the syncytiotrophoblast is partly regenerated within 48 h of culture. Conversely, the majority of villous cytotrophoblasts underlying the syncytiotrophoblast and cells of the mesenchymal core die during the first week of culture. Despite this extensive villous cell death, multilayered extravillous trophoblast progenitors in the villous tips remained viable, and explants retained the capacity to produce new extravillous trophoblast outgrowth for up to 3 weeks. We believe that this demonstrates that first trimester villi contain two distinct villous cytotrophoblast populations that are committed to differentiate either into syncytiotrophoblast or extravillous trophoblast.

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The regulation of trophoblast differentiation by oxygen in the first trimester of pregnancy

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In the first trimester of human pregnancy villous cytotrophoblasts are able to differentiate to form either the overlying syncytiotrophoblast layer or, in anchoring villi, extravillous trophoblasts which grow out from the villi and invade into the maternal decidua, acting to both physically attach the placenta to the decidua, and modify the maternal spiral arteries to sustain pregnancy. During the first 10–12 weeks of gestation, extravillous trophoblast plugs block the spiral arteries and prevent maternal blood flow entering the intervillous space, thereby creating an environment of physiological hypoxia in which placental and fetal development occur. As extravillous trophoblasts migrate away from the villi they differentiate from a proliferative to an invasive phenotype. The hypoxic environment of the first trimester is believed to play an important role in the regulation of trophoblast differentiation. However, there is currently a large body of conflicting experimental evidence concerning this topic. This review examines the experimental evidence to date on the role of oxygen in trophoblast differentiation.

Key words: differentiation/hypoxia inducible factor/hypoxia/placenta/trophoblast

Introduction

Placentaion is initiated when the blastocyst makes contact with the epithelial lining of the uterus shortly after implantation. Placental villi develop which consist of a mesenchymal core surrounded by a monolayer of mononuclear villous cytotrophoblast stem cells which either fuse to form the overlying multinucleated syncytiotrophoblast or, in anchoring villi, differentiate into extravillous trophoblasts which grow out from the villi and spread laterally around the placenta (Irving et al., 1995). As extravillous trophoblasts move away from the placenta they differentiate into an invasive phenotype in a process which is tightly regulated both spatially and temporally and is essential for the success of pregnancy. Phenotypically there are key differences between invasive extravillous trophoblasts and villous cytotrophoblasts. Such differences can be seen in the expression of cell surface molecules such as adhesion molecules, as well as in the secretion of cytokines, growth factors and proteases (Norwitz et al., 2001).

Invasive extravillous trophoblasts play an important role in adapting the decidua to sustain pregnancy. Extravillous trophoblasts invade the walls of the uterine spiral arteries and adapt these vessels into large bore conduits capable of delivering the increased blood supply required in the second and third trimesters and ensure that this blood supply is independent of maternal vasoconstriction (Robertson et al., 1967; Zhong et al., 1997). As the extravillous trophoblasts invade the spiral arteries early in pregnancy they form plugs which occlude the spiral arteries and prevent maternal blood from entering the intervillous space, thereby creating a physiological hypoxic environment (Huston and Schaaps, 1987; for review see Jaffe et al., 1997; Burton et al., 1999). From 8 to 10 weeks of gestation direct measurements of oxygen tension showed that the partial pressure of oxygen in the placenta (17.9 mm Hg) was significantly lower than the endometrium (39.6 mm Hg) (Rodesch et al., 1992). Furthermore, from 12 to 13 weeks of gestation the placental oxygen tension increased to levels which were not significantly different from the endometrium, consistent with the loosening of the trophoblast plugs at that time (Rodesch et al., 1992). More recently, Jauniaux and colleagues measured respiratory gases and acid-base values in 30 early pregnancies and confirmed that before 11 weeks of gestation the partial pressure of oxygen in the placenta was 2.5 times lower than that in the decidua (Jauniaux et al., 2001). Near the end of the first trimester the trophoblast plugs progressively loosen, exposing the developing placenta to maternal blood flow from approximately 10 weeks of gestation (Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). However, it is important to note that while there is a mounting body of evidence to support a low oxygen environment during the first trimester, not all experimental evidence supports the ability of trophoblast plugs to block maternal blood flow into the intervillous space beyond 6 weeks of gestation, with one study showing that the majority of spiral arteries are not plugged, as well as sonographic evidence that maternal blood flow into the intervillous space occurs early in the first trimester (Kuriak et al., 1993; Kuriak and Kupscie, 1997; Mecklenburg et al., 1997). Nevertheless the current
weight of evidence supports the concept that the placenta and fetus develop in a hypoxic environment in the first trimester. The early gestation placenta is poorly protected against oxidative damage, as the antioxidant enzymes copper/zinc superoxide dismutase and mitochondrial superoxide dismutase are not expressed by the syncytiotrophoblast until approximately 8-9 weeks of gestation, rendering the syncytiotrophoblast acutely sensitive to oxygen mediated damage (Watson et al., 1997). The expression of these protective enzymes increases significantly after this point as the trophoblast plugs loosen and the placenta becomes exposed to gradually increasing levels of oxygen and consequently experiences oxidative stress (Watson et al., 1997, 1998; Jauniaux et al., 2000).

To be able to respond to increasing levels of oxygen, as the maternal-placental circulation is established at the end of the first trimester and as invasive trophoblasts progress along the spiral arteries, trophoblasts must be able to accurately sense oxygen tension (Caniggia and Warton, 2002). The exact mechanism by which trophoblasts sense oxygen tension is currently unclear, however, several potential pathways have been identified. Many of these pathways utilize the formation of reactive oxygen species (ROS), but it is currently unclear whether hypoxia results in an increase or decrease in cellular levels of ROS (for review see DeMarco and Caniggia, 2002). In hypoxic conditions, trophoblast oxygen sensing mechanisms utilize several different pathways to control gene expression. These pathways often utilize redox-sensitive transcription factors, of which the hypoxia inducible factor (HIF) family are the best characterized in trophoblasts.

**The HIF family of transcription factors**

To date, three members of the HIF family have been identified, but it is possible that others exist. All the members of the HIF family consist of an inducible α subunit (HIF-α) and a constitutively expressed beta subunit (HIF-β), also known as aryl hydrocarbon receptor nuclear translocator (ARNT). Under physiologically normoxic conditions HIF-α is rapidly broken down following binding of von Hippel-Lindau tumour suppressor protein (pVHL), which targets HIF-α for degradation by the ubiquitin-proteasome pathway (Figure 1) (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000). pVHL binds to HIF-α following hydroxylation of proline residues in the oxygen-dependent degradation domain of HIF-α (Jaakkola et al., 2001; Masson et al., 2001). However, under hypoxic conditions this hydroxylation of the proline residues is blocked, resulting in stabilization and accumulation of HIF-α in the cytoplasm (Figure 1) (Ivan et al., 2001; Lande et al., 2002). The stabilized HIF-α can translocate to the nucleus where it dimerizes with HIF-β. The HIF-α-β dimer is then able to bind to DNA and induce gene expression (Wang et al., 1995). Hypoxia further enhances the activity of HIF-α by promoting the ability of the HIF-α C-terminal transactivation domain (CAD) to interact with coactivators such as p300 and thereby amplify HIF-α induced gene transcription (Ema et al., 1999).

![Figure 1. Regulation of hypoxia inducible factor-1 (HIF-1). Under hypoxic conditions, HIF-1α is phosphorylated allowing it to bind HIF-β and co-factors such as p300 to induce gene transcription. The phosphorylation of HIF-1α is not solely dependent on hypoxia, and the binding of growth factors to extracellular receptors can affect this process. Under normoxic conditions, HIF-1α is targeted for degradation along the ubiquitin-proteasome pathway by the binding of pVHL, preventing the formation of active HIF-1. Hypoxia inhibits pVHL and factor inhibiting HIF-1 (FIH-1) allowing protein expression and transcriptional activity of HIF-1α. HIF is able to be regulated independently of oxygen by the binding of growth factors to receptor tyrosine kinases which can firstly induce translation of HIF-1α to overcome the oxygen sensor mediated HIF-1α degradation and secondly enhance the transcriptional activity of active HIF by phosphorylation of the p300 co-activator. HRE, hormone responsive element; IGF, insulin-like growth factor; MAPK, mitogen-activated protein kinase; TNF-α, tumour necrosis factor-α.](image-url)
HIF-1

The predominant member of the HIF family is HIF-1, which has been shown to regulate the expression of more than 60 genes in a variety of cell types (Semenza, 2003). In the placenta in vivo, HIF-1α has been shown to be strongly expressed from 5 weeks of gestation, predominantly in the extravillous trophoblast, syncytiotrophoblast and villous cytotrophoblast (Caniggia et al., 2000b; Rajakumar and Conrad, 2000). However, expression decreases at around 9 weeks of gestation, and by 12 weeks of gestation immunoreactivity for HIF-1α is weak or absent (Caniggia et al., 2000b; Caniggia and Winter, 2002).

HIF-2α and -3

HIF-2α and -3 arise from combinations of HIF-2α and -3α subunits, respectively, with the common HIF-β chain. HIF-2α is expressed in the syncytiotrophoblast, cytotrophoblasts and mesenchymal cells of the first trimester villous placenta (Rajakumar and Conrad, 2000). HIF-2α mRNA increases significantly with advancing gestational age, but conversely HIF-2α protein decreases with gestational age, indicating that as for the degradation of HIF-1α by pVHL, the main point of regulation for HIF-2α is post-transcriptional (Rajakumar and Conrad, 2000). In situ, pVHL is expressed in villous cytotrophoblast cells and sites of invasive trophoblast column initiation, corresponding to cytoplasmic HIF-2α staining (Gembacev et al., 2001). Confusingly, it has been reported that as trophoblasts invade the decidua, pVHL is down-regulated and HIF-2α is localized to the nucleus (Gembacev et al., 2001). The up-regulation of trophoblast pVHL in response to hypoxia has been confirmed in vitro (Gembacev et al., 2001). Anchoring villi with attached trophoblast columns show strong staining for pVHL when cultured in 2% oxygen, whereas in 10% or 20% oxygen staining for pVHL is weak or absent (Gembacev et al., 2001). These results are contrary to expectation as higher oxygen conditions are proposed to result in the degradation of HIF-2α by pVHL, therefore pVHL would be expected to be down-regulated in hypoxic conditions. However, it seems likely that the ratio of HIF-2α to pVHL is more important than the absolute levels of either protein in determining a cell’s response to low oxygen. HIF-2α would also be expected to be targeted for degradation when oxygen tension increased, not localized to the nucleus as observed in this study (Gembacev et al., 2001). The function of HIF-2α in the placenta is yet to be elucidated.

Human HIF-3α was first identified in 2001, however, due to its placental expression pattern and functional role have not been reported (Hara et al., 2001).

Regulation of HIF

Regulation of trophoblast differentiation by oxygen

The protein expression and transcriptional activity of HIF-1α is able to be regulated independently of hypoxia through the phosphatidylinositol 3 kinase (PI3K) and/or mitogen-activated protein kinase (MAPK) signal transduction pathways allowing cell specific fine tuning of the hypoxic response (Figure 1) (Wenger, 2002; Lee et al., 2004).

The transcriptional activity of HIF-1α is regulated by both transcription (Figure 1) (Conrad et al., 1999; Richard et al., 1999; Jiang et al., 2001; Fukuda et al., 2002). The MAPK pathway, in particular, is known to play an important role in the signal transduction of a range of cellular responses including proliferation, differentiation and stress responses (Kita et al., 2003). In human trophoblasts, the PI3K signalling pathway is activated by interleukin-12, whereas the MAPK signalling pathway is known to be involved in the effects of insulin, IGF-II, insulin-like growth factor binding protein-1 (IGFBP-1), leptin, endothelin-1, GnRH and EGF (Kang et al., 2000; Gleeson et al., 2001; McAdam et al., 2001; Kong et al., 2002; Brincker et al., 2003; Chakraborty et al., 2003; Mockova et al., 2003). The MAPK pathway can also be activated by the formation of ROS in hypoxic conditions (Katul et al., 2002).

Finally, the transcriptional activity of HIF-1α can be regulated by several different transcription factors, including those involved in the response to hypoxia. Several other transcription factors involved in trophoblast differentiation are responsive to hypoxia. The transcription factors Id1, Mash2 and the hexin-loop-helix transcription factors upstream stimulatory factor-1 and -2 (USF1 and USF2) which mediate the effects of Mash2 are all up-regulated in 2% oxygen in comparison to 20% oxygen (Jiang et al., 2000; Jiang and Mendelson, 2003). The up-regulation of Mash2, USF1 and USF2 may inhibit cytotrophoblast fusion into syncytiotrophoblast (Jiang et al., 2000; Jiang and Mendelson, 2003).

The regulation of intracellular Ca²⁺ is believed to act as an HIF-1-independent signalling pathway, which involves the transcription factor activator protein-1 (AP-1), with cooperation between the HIF-1 and AP-1 pathways allowing fine regulation of hypoxic gene expression (Laderoute et al., 2002; Salmanik et al., 2002) (Figure 2). AP-1 is a dimeric transcription factor composed of the products of the Jun and Fos proto-oncogenes (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) (Dykour et al., 1990). AP-1 transcription factors are believed to play an important role in trophoblast differentiation. The role of AP-1 transcription factor expression is limited, however, extravillous trophoblasts express c-Jun, JunB, c-Fos, FosB and Fra-2 both in the first trimester and...
Figure 2. Regulation of hypoxia-inducible [von-hypoxia inducible factor (HIF)] transcription factors. Hypoxia results in the up-regulation of many transcription factors including the Jun subunit of AP-1 transcription factors, as well as Id1, Mash2 and upstream stimulatory factor-1 and -2 (USF1 and USF2). The Jun proto-oncogenes are able to bind to other Jun subunits or to Fos subunits to form AP-1 transcription factors which are then able to bind to one of three AP-1 transcription factor binding sites in the promoter region of hypoxia-inducible target genes and induce transcription. Jun can also be up-regulated independently of hypoxia by increased cellular calcium levels. Id1 and Mash2 can bind directly to the promoter region of target genes and induce transcription. The up-regulation of Mash2 also results in the up regulation of USF1 and USF2 which further induce transcription of hypoxia regulated genes by binding to E boxes in the promoter region of the target gene.

later in gestation (Bamberger et al., 2004). To date, no data on trophoblast intracellular calcium elevation or the role of the AP-1 pathway in mediating the effects of hypoxia in trophoblasts has been published. However, it is feasible that, as hypoxia is able to influence the AP-1 pathway in other cell types, this pathway may play a role in the response of trophoblasts to hypoxia (Schorpp-Kistner et al., 1999).

The trophoblast response to hypoxia in the first trimester of pregnancy

Transcription factors facilitate the expression of a wide range of genes in response to hypoxia in the first trimester of pregnancy. Gene responses to hypoxia can be broadly divided into two groups—those that promote cell survival by increasing oxygen delivery, decreasing oxygen consumption and adapting cellular metabolic responses and are therefore a common response to hypoxia in many tissues and those that play a specific role in the regulation of implantation and placentation. Changes in gene expression in hypoxia that promote trophoblast survival include increased glucose consumption and the expression of proteins involved in glycolysis (Hoang et al., 2001; Baumann et al., 2002). The remainder of this review will focus specifically on the second group and how hypoxia regulates trophoblast differentiation in the first trimester.

The effect of low oxygen on trophoblast differentiation

In vivo, only the extravillous trophoblasts most proximal to the villi proliferate (Irving et al., 1995; Vivovac et al., 1995). As extravillous trophoblasts migrate away from the villi and invade into the maternal decidua they progressively develop an invasive phenotype and are no longer able to proliferate (Gernaey et al., 1997, 2000). There are two opposing schools of thought on the effect of hypoxia on trophoblast differentiation in the first trimester of human pregnancy. Evidence exists to demonstrate that hypoxia promotes a proliferative trophoblast phenotype, which would create a large pool of trophoblasts, thereby providing sufficient numbers to invade into the maternal decidua. However, contradictory results have also shown hypoxia to promote an invasive trophoblast phenotype which may be important in achieving sufficient depth and extent of trophoblast invasion.

Evidence for low oxygen promoting a proliferative phenotype in villous trophoblast

Several different lines of evidence support the hypothesis that hypoxia promotes trophoblast proliferation. In vivo, there is a high level of mitoses in the villous cytotrophoblast between 6 and 10 weeks of gestation, but the mitotic index decreases significantly between 10 and 12 weeks of gestation when the placenta is exposed to maternal blood (Tedde and Tedde Piras, 1978). This observation is supported by in vitro evidence. Firstly, HTR-8/SVneo cells, a human first trimester cytotrophoblast cell line, show both increased proliferation and reduced invasion through Matrigel when cultured in 2% oxygen conditions (Kilburn et al., 2000). Secondly, isolated first trimester cytotrophoblasts show increased rates of DNA synthesis in 2% oxygen in comparison to 20% oxygen (Jiang et al., 2000). Thirdly, in vitro the ratio of cytotrophoblast : syncytiotrophoblast nuclei abruptly declines in placentae over 8 weeks of gestation despite the number of cytotrophoblast nuclei per unit area remaining constant suggesting that
cytotrophoblast proliferation is greater in the hypoxic conditions of early pregnancy (Bose et al., 2003). Finally, in comparison to explants cultured in 20% oxygen, first trimester villous explants cultured in 2 or 3% oxygen show increased 5-bromo-2'-deoxyuridine (BrdU) incorporation, increased extravillous trophoblast outgrowth and an increase in the total number of cells in this outgrowth (Gembacev et al., 1997; Caniggia et al., 2000b).

In support of these results, first trimester villous explants cultured in 1 and 5% oxygen formed 55 and 40% more outgrowths, respectively, than explants cultured in 20% oxygen (Sferruzzi-Perri and Roberts, 2003). In contrast, our group has observed a 27% decrease in the number of extravillous trophoblast outgrowths formed from first trimester villous explants cultured in 1.5% oxygen in comparison to those cultured in 8% oxygen (James et al., 2004). Furthermore, in our study, extravillous trophoblast outgrowths formed under hypoxic conditions contained fewer cells than those produced in 8% oxygen (James et al., 2004).

Hypoxia has also been shown to reduce the invasive capacity of trophoblasts and the expression of molecules associated with an invasive trophoblast phenotype such as α5 integrin and matrix metalloproteinase-2 (MMP-2) (Gembacev et al., 1997; Caniggia et al., 2000a; Kilburn et al., 2000; Crocker et al., 2003). Culture in 3% oxygen also reduces the invasion of isolated cytotrophoblasts from the vessel lumens into the walls of dissected spiral arteries in comparison to culture in 20% oxygen (Crocker et al., 2003)

Further indirect evidence for the stimulation of a proliferative trophoblast phenotype in response to hypoxia is provided by the regulation of key cytokines that promote this phenotype. Transforming growth factor β (TGFβ) acts under the control of HIF-1α to inhibit trophoblast invasion (Caniggia et al., 2000b; Schaller et al., 2003; Nishi et al., 2004). TGFβ expression in placental villi has been reported to increase from approximately 6 to 9 weeks of gestation, with a failure in its down-regulation around 9 weeks of gestation reported to be associated with shallow trophoblast invasion, which may predispose the pregnancy to pre-eclampsia (Caniggia et al., 1999, 2000a). Therefore, induction of HIF-1α by hypoxia in the first trimester may up-regulate TGFβ expression, inhibiting trophoblast differentiation to an invasive phenotype (Caniggia et al., 2000a).

However, these temporal changes in TGFβ expression were not detected by Simpson and colleagues, who could only detect low levels of TGFβ immunostaining between 7 and 19 weeks of gestation (Simpson et al., 2002).

IGF-II has also been identified as a cytokine which is able to mediate the effects of hypoxia on extravillous outgrowth (Sferruzzi-Perri and Roberts, 2003). Addition of exogenous IGF-II to first trimester villous explant cultures increases the formation of extravillous trophoblast outgrowth in 20% oxygen conditions, but not in 1 or 5% oxygen conditions in which outgrowth production is elevated independent of exogenous IGF-II (Sferruzzi-Perri and Roberts, 2005).

### Evidence for low oxygen promoting an invasive trophoblast phenotype

In contrast to the above discussion, a smaller body of indirect evidence exists that suggests that hypoxia may induce extravillous trophoblast differentiation into an invasive trophoblast phenotype. One mechanism of trophoblast invasion involves urokinase-type plasminogen activator (uPA), which is secreted as an inactive pro-enzyme that is activated upon binding to its highly specific receptor (Blasi, 1993). Receptor bound uPA is able to convert plasminogen into plasmin, which is then able to degrade several ECM components, activating growth factors and latent metalloproteases required for invasion (Saksela, 1985; Petersen et al., 1988; Mayer, 1990). In direct contrast with the results of Kilburn and colleagues discussed previously, Graham and colleagues found that culture of HTR-8/SVneo trophoblast cells in 1% oxygen increased the invasion of these cells through Matrigel in comparison to culture in 20% oxygen, by up-regulating uPA receptors (Graham et al., 1998). The uPA system is regulated by PAI-1 and PAI-2, which inhibit both free and bound uPA by forming irreversible covalent complexes. HTR-8/SVneo cells cultured in hypoxia show elevated PAI-1 expression (Fitzpatrick and Graham, 1998). Therefore, Graham and colleagues propose a model whereby hypoxia stimulates trophoblast invasion by increasing uPA receptor expression at the leading edge of the invading cell, as well as stimulating the secretion of PAI-1 at the receding edge where proteolytic activity is no longer required but detachment of the cell is needed for migration (Graham et al., 2000).

Leptin is a hormone primarily produced in humans by adipocytes and has a role in the regulation of food intake and energy expenditure. However significant amounts of leptin are also produced by the placenta during pregnancy, with levels peaking in the second trimester (Mozosaki et al., 1997). Leptin is expressed homogenously throughout extravillous cell columns, the leptin receptor is expressed in a clear gradient along the extravillous trophoblast columns with the strongest expression at the distal cells of the columns (Castellucci et al., 2000). Accordingly the binding of leptin to its receptor is believed to stimulate an invasive trophoblast phenotype (Castellucci et al., 2000).

In vitro, hypoxia activates the human leptin gene promoter through HIF-1 in BeWo choriocarcinoma cells (Grossfeld et al., 2002). Therefore, leptin may provide another mechanism by which hypoxia could stimulate an invasive trophoblast phenotype. However, the role of hypoxia in the stimulation of leptin production and activity in the first trimester of human pregnancy has yet to be elucidated.

Alterations in the trophoblast adhesion molecule repertoire and production of extracellular matrix components by hypoxia have been linked with extravillous trophoblast differentiation into an invasive phenotype. HTR-8/SVneo cells cultured in hypoxic conditions show increased trophoblast fibronectin production, but decreased expression of α5 integrin which forms part of the α5β1 integrin fibronectin receptor and consequently decreased trophoblast adhesion to fibronectin (Lash et al., 2001; Chen and Aplin, 2003). It is possible that increased trophoblast fibronectin production is an indirect result of the acidic environment that hypoxia creates (Gaus et al., 2002). However, despite the above evidence being used by several authors as support for an invasive phenotype, fibronectin blocking antibodies do not affect trophoblast invasion in vitro, and consequently α5β1 integrin is not believed to play a significant role in trophoblast invasion (Damsky et al., 1994). The uPA receptor may also interact directly with specific integrins, such as β1 integrin, to modulate trophoblast adhesion to, and migration towards, ECM vitronectin (Wei et al., 1996; Lash et al., 2001). However, PAI-1 is also able to act as an antagonist by binding to vitronectin and preventing its association with the uPA receptor (Lash et al., 2001).
Does the physiological hypoxia of early pregnancy promote an invasive or a proliferative trophoblast phenotype?

The confusion as to the effect of hypoxia on trophoblast differentiation in the first trimester of human pregnancy is confounded by the fact that the cellular response to hypoxia is not an isolated pathway, but a combination of multiple sensing mechanisms, transcription factors, oxidative stress responses and cytokine production, not to mention a large number of discordant results in the literature. Many of the studies providing evidence for hypoxia promoting an invasive trophoblast phenotype use the extravillous trophoblast hybrid cell line HTR-8/SVneo, as a substitute for extravillous trophoblasts which are difficult to propagate in culture, and whether this cell line truly reflects the behaviour of normal trophoblasts is open to debate. The use of explant cultures to study extravillous trophoblast differentiation provides significant advantages in that they allow outgrowth in a manner similar to that which occurs in vivo, but results from individual explants tend to be variable and therefore a high number of replicates are required to create meaningful data. It is interesting to note that the gene expression profile of first trimester villous explants cultured in 3% oxygen is remarkably similar to that of term placenta from high-altitude and pre-eclamptic pregnancies indicating that, although a low oxygen environment is the physiological norm in the first trimester, this model may provide insights into how a decreased oxygen supply may affect the placenta later in gestation (Soleymannia et al., 2005). The interpretation of what extravillous trophoblast outgrowth from explants signifies in different explant models is also often unclear, with some reports concluding that this is indicative of extravillous trophoblast proliferation and clearly separate it from extravillous trophoblast invasion (Czegle et al., 2000b), whereas others conclude that extravillous trophoblast outgrowth represents trophoblast invasion (Irving et al., 1995; James et al., 2004). Finally, as the acquisition of an invasive phenotype is a progressive phenomenon we should not assume that the proliferative and invasive trophoblast phenotypes are necessarily mutually exclusive, and it is possible that the effect of hypoxia on trophoblast phenotype may vary with the specific properties of various cell groups.

It is important to note that many of the above experiments used physiologically suprOXic conditions of 2% oxygen (pO₂ = 140 mm Hg) as a control. However, once the maternofetal circulation is established the blood in the intervillous space is a mixture of arterial and venous blood, and blood sampled from the intervillous space at term has a pO₂ of 40 mm Hg (Howard et al., 1961). Therefore, a more physiologically relevant control concentration of around 6-8% oxygen may more accurately represent conditions in vivo once the maternal circulation in the intervillous space is established. Furthermore, the range of experimental oxygen concentrations (from 1 to 3% oxygen) used to represent hypoxia may also contribute to the experimental differences observed. Finally, the gestation and mode of collection of placenta may also be important as exposure to maternal blood or significant time delay at the time of tissue collection leading to oxidative stress is able to affect immunoreactivity of not only HIF-1α, but the induction of downstream factors such as TGFβ3 (Dyson et al., 2003).

In conclusion, trophoblast differentiation is essential for the success of human pregnancy, and despite some conflicting experimental evidence, hypoxia appears to play a vital role in regulating trophoblast differentiation in the first trimester. The regulation of trophoblast differentiation by hypoxia is a result of complex interactions between factors associated with oxidative stress, oxygen sensing mechanisms and the release of inflammatory cytokines. Therefore, aberrations in any one of these factors, along with the temporal and spatial regulation of blood flow in the intervillous space has the potential to result in altered gene expression and trophoblast phenotype.

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The effects of oxygen concentration and gestational age on extravillous trophoblast outgrowth in a human first trimester villous explant model

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BACKGROUND: In the first trimester of human pregnancy, extravillous trophoblasts from placental villi invade the decidua temporarily occluding the spiral arteries, preventing maternal blood flow and creating a low-oxygen environment, which is believed to play an important role in the regulation of extravillous trophoblast outgrowth. This work aimed to quantify the effects of gestational age and oxygen concentration on extravillous trophoblast outgrowth. METHODS: A quantitative first trimester villous explant model was used to measure the frequency and area of extravillous trophoblast outgrowths from villi grown in 1.5 or 8% oxygen. RESULTS: The percentage of explants producing outgrowth declined independently of oxygen concentration as gestation increased from 8 to 12 weeks. Culture in 1.5% oxygen significantly reduced the frequency and area of outgrowths in comparison with 8% oxygen. HLA-G and α₅ integrin were both expressed throughout outgrowths, with no difference in the expression between oxygen concentrations. Gestation influenced the response of explants to oxygen, with a significant differential response to oxygen concentration in placentae under 11 weeks of gestation, whereas in villi from placentae of 11 or 12 weeks, no differential response was observed. CONCLUSIONS: In the first trimester, oxygen and gestational age both regulate extravillous trophoblast outgrowth.

Key words: cytotrophoblast differentiation/extravillous trophoblast/hypoxia/placenta

Introduction

In the first trimester of human pregnancy, placental villi consist of a core of mesenchymal cells surrounded by a monolayer of mononuclear cytotrophoblast cells which either fuse to form the overlying multinucleated syncytiotrophoblast or, in anchoring villi, differentiate into extravillous trophoblasts that grow out from the villi in columns and invade into the maternal decidua (Boyd and Hamilton, 1970). As the extravillous trophoblasts in the columns move away from the anchoring villi, they also spread laterally around the implantation site and invade the maternal spiral arteries (Brosens et al., 1967; Kam et al., 1999). The invasion of the spiral arteries by extravillous trophoblasts leads to the transformation of these vessels into large-bore conduits that allow the increased maternal blood flow that is required for fetal growth. The transformation of the spiral arteries by extravillous trophoblasts is called the ‘physiological changes of pregnancy’, and inadequate physiological changes of pregnancy are found in pregnancies complicated by pre-eclampsia and intrauterine growth restriction (Brosens et al., 1967; Khong et al., 1986).

It is now apparent that from at least 5–10 weeks of gestation, extravillous trophoblasts form plugs in the maternal spiral arteries occluding the flow of maternal blood to the intervillous space (Hustin and Schaaps, 1987; Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). Thus, for the majority of the first trimester, the placenta develops in conditions of physiological hypoxia in which the local oxygen concentration is as low as 1–2% (Rodesch et al., 1992). The trophoblast plugs are believed to progressively disperse from 10 to 12 weeks of gestation, allowing maternal blood to flow into the intervillous space (Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). It is believed that the low-oxygen conditions during the first 10–12 weeks of pregnancy regulate placental development and extravillous trophoblast outgrowth (reviewed in James et al., 2005a).

In this study, we have quantified extravillous trophoblast outgrowth from large numbers of cultured first trimester villous explants to determine the effects on extravillous trophoblast outgrowth of (i) gestational age and (ii) oxygen concentration and (iii) whether these effects are interdependent.

Materials and methods

This study was approved by the Auckland Regional Ethics Committee, and all tissue samples were obtained with informed consent.
Explant culture and image analysis
First trimester placenta were obtained following elective surgical termination of pregnancy (TOP) and washed gently in phosphate-buffered saline (PBS, pH 7.4). The gestational age, based on crown-rump length, and fetal viability of all pregnancies before TOP were confirmed by ultrasound assessment. Villous explants were cultured as described previously (James et al., 2005b). Briefly, Matrigel (Bectin Dickinson, Sydney, Australia) was diluted to 1:10 in Dulbecco’s modified Eagle’s medium salts at 4°C (DMEM/F12) (Life Technologies, Auckland, New Zealand) and used to coat wells of sterile 96-well culture plates (Falcon, Sydney, Australia) with an extremely thin layer of Matrigel. Villous tips of 8 mg wet weight were gently teased from the placentae, and randomly selected explants were placed in the centre of each well. The villous explants were incubated at 37°C for 5 min and then 150 µl/well of complete trophoblast medium [DMEM/F12 containing 10% fetal bovine serum (FBS), 5 µg/ml of epidermal growth factor, 5 µg/ml of insulin, 10 µg/ml of transferrin, 100 µg/ml of streptomycin, sodium selenite 20 nM, 400 U/ml of hCG and 100 U/ml of penicillin] was added. The plate was then centrifuged at 210 g for 1 min. At least 75 explants were prepared from each of the five placenta of 8, 9, 10, 11 and 12 weeks of gestation and cultured in sealed plastic blood bags (Jackson Allison, Auckland, New Zealand) in 8% O₂ with 5% CO₂ in N₂. An additional culture of at least 75 explants from each of these 25 placenta was prepared in the same manner but cultured in blood bags containing 1.5% O₂ with 5% CO₂ in N₂. The bags were flushed with fresh gas mix daily, and the oxygen concentration of each bag was confirmed at the end of the culture period by syringe gas cylinder analysis (Bacharach, Pittsburgh, PA, USA). On day 5 of culture, two-dimensional (2D) outgrowth of trophoblasts from the explants across the thin layer of Matrigel was observed by phase-contrast microscopy using an inverted microscope (Nikon EL WD 0.3 Phase Contrast microscope, Nikon, Tokyo, Japan), and the frequency of extravillous outgrowth was determined by recording those explants which produced extravillous (trophoblast) outgrowth in relation to the total explants cultured. The area of extravillous trophoblast outgrowth was determined by photographing outgrowths using a Nikon Coolpix 990 digital camera (Nikon). Overlapping images were aligned using Adobe Photoshop 5.0, and the areas of trophoblast outgrowth were measured by manual tracing using Image J software.

Statistical analysis
Data analysis was performed using Microsoft Excel 2002 and WinSTAT for Excel. The statistical significance of the relationship between gestational age and the frequency of outgrowth production was determined by analysis of variance (ANOVA). A dependent Student’s t-test was used to calculate the statistical significance of the outgrowth frequency data. The data generated from the measurement of outgrowth areas were not normally distributed; therefore, a Mann-Whitney U-test was used to calculate its statistical significance.

The differential response of explants to culture in 1.5 and 8% oxygen was defined using the formula: percentage of explants producing outgrowth in 8% oxygen - percentage of explants producing outgrowth in 1.5% oxygen. The significance of the differential response to oxygen was analysed by a t-test. The significance of this data was also determined by gestational age using a single factor ANOVA.

In situ immunohistochemistry
Media were removed from explants which were retained in the culture plates and washed with PBS and then fixed in 100 µl of methanol for 10 min. Wells were washed once with PBS (pH 7.4) containing 0.05% Tween-20 (PBS-Tween); then non-specific binding was blocked by the addition of 100 µl of 10% normal goat serum (NGS) in PBS-Tween (Life Technologies) for 10 min at room temperature. Wells were then washed three times with PBS-Tween. One hundred microlitres of 1:66 αβ integrin (Sigma, Castle Hill, NSW, Australia) and 1:200 HLA-G diluted in 10% NGS in PBS-Tween was added for 1 h at room temperature (Sapphire Biosciences, Redfern, NSW, Australia). One well stained with 1:500 cytokeratin 7 was used as a positive control, and wells stained with 1:500 vimentin and 10% NGS in PBS-Tween containing no antibody were used as negative controls. Each antibody was used on explant cultures from 1.5 and 8% oxygen cultures from three placenta of the same gestational age, such that in total 36 outgrowths were stained for αβ integrin expression and 26 outgrowths were stained for HLA-G expression. Wells were then washed three times with PBS-Tween, and endogenous peroxidase activity was quenched by the addition of 50 µl of 3% H₂O₂ in methanol for 5 min. Wells were then washed three times with PBS-Tween. A Zymed Histostain-Plus Kit (Zymed, San Francisco, CA, USA) containing biotinylated secondary antibody and enzyme conjugate was used according to the manufacturer’s instructions. Wells were washed three times with PBS-Tween, and staining was developed with 3,3'-diaminobenzidine (DAB) (0.1% w/v DAB, 0.001% H₂O₂ in PBS) for 20 min. Wells were then washed with de-ionized water. One hundred microlitres of haemotoxylin nuclear stain (Surgepath, Australia Laboratory Services, Auckland, New Zealand) was added to each well for 4 min. Wells were washed with tap water. Outgrowth staining was observed immediately using an inverted microscope (Nikon EL WD 0.3 Phase Contrast microscope, Nikon) and digitally photographed using a Nikon Coolpix 990 digital camera (Nikon).

Results
Gestational age affects the number of villi producing extravillous trophoblast outgrowth but not the size of the outgrowths
Of the 3963 explants cultured in this study, 555 (14%) produced extravillous trophoblast outgrowths. A further 13.8% of explants floated in the culture media, and because they were not in contact with the Matrigel surface, these explants were excluded from the analysis. The percentage of explants from each placenta that produced extravillous trophoblast outgrowth declined progressively with increasing gestational age of the tissue, independent of oxygen concentration (F = 0.012 and 0.001 in 1.5 and 8% oxygen cultures, respectively) (Figure 1). However, the gestational age of the tissue did not affect the area of extravillous trophoblast outgrowths produced by individual villi.

The effect of oxygen concentration on extravillous trophoblast outgrowth
To determine the effect of oxygen concentration on the number of explants producing extravillous trophoblast outgrowths, we compared the percentage of explants regardless of the gestational age that produced outgrowth when cultured in either 1.5 or 8% oxygen. Significantly fewer (P = 0.0001) explants produced extravillous trophoblast outgrowths in 1.5% oxygen (14%, n = 239/1713) than in 8% oxygen (18.6%, n = 317/1702) (Figure 2).

To determine the effect of oxygen concentration on the size of extravillous trophoblast outgrowths, we compared the mean area of outgrowth from explants cultured in either 1.5 or 8% oxygen (Figure 3). The mean area of extravillous trophoblast outgrowths produced in 1.5% oxygen (0.94 mm², n = 238)

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Figure 1. Scatterplot demonstrating the percentage of explants in 8% (■) and 1.5% (●) oxygen conditions that produced extravillous trophoblast outgrowth in relation to gestational age. The percentage of explants from each placenta which produced extravillous trophoblast outgrowth in each culture is represented by one data point on the graph. The percentage of explants that produce extravillous trophoblast outgrowth declined significantly with increasing gestational age in both 1.5% ($P = 0.012$) and 8% ($P = 0.001$) oxygen conditions.

Figure 2. Histogram demonstrating the mean percentage of first trimester villous explants that produced extravillous trophoblast outgrowth in 1.5% oxygen (14%) was significantly less ($P < 0.001^*$) than that produced in 8% oxygen (18.6%).

was significantly less ($P = 0.028$) than in 8% oxygen (1.26 mm$^3$, $n = 317$).

To determine whether oxygen concentration altered the phenotype of extravillous trophoblasts, we stained outgrowths from explants cultured in 1.5 or 8% oxygen with antibodies reactive with $\alpha_1$ integrin and HLA-G. $\alpha_1$ integrin and HLA-G were expressed on extravillous trophoblasts throughout the outgrowths in both 1.5 and 8% oxygen cultures, and there were no apparent differences in the expression of these proteins by extravillous trophoblasts grown in 1.5 or 8% oxygen (Figure 4).

The relationship between gestational age and the effect of oxygen concentration on the formation of extravillous trophoblast outgrowth

To examine whether the effect of oxygen concentration applied equally to placenta of different gestations, we examined the differential response of villous explants to oxygen concentration by determining the difference in the percentage of explants that produced outgrowths using matched cultures of explants from the same placenta. There was a significant difference ($P = 0.0005$) between the clear differential response of explants from the same placenta to oxygen concentration for placenta under 11 weeks of gestation (8.4%, $n = 15$ placenta) and the negligible differential response observed from explants from placenta of 11 weeks of gestation and greater (0.79%, $n = 10$ placenta) (Figure 5). The alteration in response to oxygen concentration with gestational age was not a gradual trend but rather appeared as a discrete change that occurred at 11 weeks of gestation (Figure 5).

Discussion

The low-oxygen environment in which placental development occurs in the first trimester of human pregnancy is believed to be important in controlling extravillous trophoblast outgrowth by directing the tightly regulated processes of trophoblast differentiation and invasion (Caniggia and Winter, 2002). In this study, we have utilized a 2D model of extravillous trophoblast outgrowth from first trimester villous explants to examine the
Figure 4. In situ immunohistochemistry of extravillous trophoblast outgrowth across the Matrigel-coated surface of a 96-well plate after 5 days of culture. Extravillous trophoblast outgrowths are counterstained with haematoxylin. HLA-G was expressed throughout the outgrowth, and no alterations in the staining pattern or intensity were observed between cultures in 1.5% (A) and 8% (B) oxygen. Likewise, α5 integrin was expressed throughout the outgrowth, and its expression was not different in cultures grown in 1.5% (C) and 8% (D) oxygen. No staining was observed with isotype-matched antibodies reactive with vimentin (E). Staining with the positive control antibody cytokeratin 7 was observed throughout the outgrowth (F). Panels A, B, E and F are of outgrowths from villous tissue of 10 weeks of gestation, whereas panels C and D are of outgrowths from villous tissue of 9 weeks of gestation.
effects of gestation and oxygen concentration on extravillous trophoblast outgrowth from villi of 8–12 weeks of gestation.

The explant model we employ allows us to examine two separate features of extravillous trophoblast development. First, we do not select villous tips that have extravillous columns as some other researchers do (Genbacev et al., 1997; Aplin et al., 1999; Caniggia et al., 2000), but rather we dissect placenta into small explants of ~8 mg which are randomly selected for culture. Because of the random nature of selection and the large number of explants cultured, a large proportion of the villi from each placenta are used, providing a good representation of the placenta as a whole. This technique allows us to examine the number of villous tips in a placenta that are capable of producing extravillous trophoblast outgrowth. Second, our explants are cultured on an extremely thin layer of Matrigel, which results in 2D extravillous trophoblast outgrowth that is readily and accurately quantifiable.

It is well known that the ratio of floating to anchoring villi increases as gestation progresses, particularly in the second and third trimesters, but our work shows that even during the last 5 weeks of the first trimester, the gestational age of placenta is important when considering extravillous trophoblast outgrowth and the response of explants to oxygen concentration. Independent of oxygen concentration, the percentage of randomly selected explants that produce extravillous trophoblast outgrowth declined progressively with the increasing gestational age of the tissue. It is widely believed that all villous cytotrophoblasts from first trimester placentae are bipotent and can form either syncytiotrophoblast or extravillous trophoblasts. In line with this belief, it has been suggested recently that all villi in the first trimester placenta are capable of forming into anchoring villi and producing extravillous trophoblasts (Bacevky et al., 2005). In contrast, as extravillous trophoblast outgrowth only occurs at the tips of the villi, it has previously been suggested that the distal tips of villi have a unique potential, which may or may not be inherent to the trophoblast at these sites (Aplin et al., 1998). Our results using very large numbers of explants expand on this suggestion by demonstrating that not all villi have the potential to become anchoring villi but rather that the numbers of villi with the potential to develop into anchoring villi decrease as the placenta expands with increasing gestational age. This study shows that as gestation progresses in the first trimester, more floating villi are formed, but these are intrinsically different to anchoring villi and as we have previously suggested, they do not have the potential to produce extravillous trophoblasts (James et al., 2005b).

In this study, villous explants produced significantly fewer extravillous trophoblast outgrowths in 1.5% oxygen than in 8% oxygen. These results are in contrast with those obtained in some other models. Others have shown that in comparison with explants cultured in 20% oxygen, first trimester villous explants of 5–8 weeks of gestation cultured under low oxygen show increased BrdU (thymidine analogue) incorporation, an increase in budding and extravillous trophoblast outgrowth from the tips of villi and an increase in the total number of cells in this outgrowth (Genbacev et al., 1997; Caniggia et al., 2000). However, the reported ‘increase’ in budding and outgrowth does not appear to have been quantified (Caniggia et al., 2000), whereas in our model, the size and frequency of extravillous trophoblast outgrowth were accurately quantified. The difference between our results and those of Caniggia et al. (2000) may also reflect the different gestational ages of the placentae studied, because this study has shown gestational age to be an important determinant of extravillous trophoblast outgrowth.
Alternatively, the differences between our results and those of Caniggia et al. may be explained because Caniggia et al. (2000) used physiologically superoxide 20% oxygen (pO₂ = 140 mmHg) as their control. Once the maternal circulation is established, the blood in the intervillous space is a mixture of arterial and venous blood and blood sampled from the intervillous space at term has a pO₂ of 40 mmHg (Howard et al., 1961). Therefore, comparative concentrations of around 6–8% oxygen as used in our study may represent conditions in vivo once the maternal circulation in the intervillous space is established more accurately than 20% oxygen. Finally, the differences between our results and those of others might be explained by the small numbers of explants studied in other reports, because there tends to be considerable variation in the behaviour of individual explants. Indeed, a study by Newby et al. (2005) in which the authors were unable to observe any significant effect of oxygen on extravillous trophoblast outgrowth from villous explants highlights the difficulties of using a model in which both the nature of the primary tissue used and the experimental outcomes show a high level of variation. In this study, we have investigated a substantially larger number of explants and placenta than others to date (3603 explants), thereby allowing us to be confident that we have accurately represented villi from the whole placenta.

The decrease in extravillous trophoblast outgrowth we have shown with respect to both frequency and size in 1.5% oxygen compared with 8% oxygen could arise from four different scenarios.

(i) Only the extravillous trophoblasts proximal to villi proliferate and are able to drive outgrowth expansion (Vivovac et al., 1995; Caniggia et al., 1997; Korhonen and Virtanen, 1997). Therefore, low-oxygen conditions may inhibit the proliferation of extravillous trophoblasts in the proximal column, thereby reducing the pool of trophoblasts able to form extravillous trophoblast outgrowth and the rate of outgrowth expansion.

(ii) It is possible that 1.5% oxygen conditions favour the proliferation of the progenitors of extravillous trophoblasts in the villi over the migration of those cells into extravillous trophoblast columns.

(iii) The smaller area of outgrowth observed in 1.5% oxygen conditions may represent a greater proportion of explants that did not produce outgrowth until later in the culture period.

(iv) Culture in 1.5% oxygen may affect the viability of extravillous trophoblast progenitors or the extravillous trophoblasts themselves, thereby reducing the number of cells able to contribute to outgrowth formation and therefore the number and size of outgrowths observed.

At present, using this model, we have been unable to distinguish between these four possible explanations.

In vivo HLA-G expression is up-regulated as extravillous trophoblasts move away from the villi in proximal columns (Shorter et al., 1992; McMaster et al., 1995), and a previous study has reported the regulation of HLA-G by oxygen in the transformed trophoblast cell line HTR-8/SVneo (Kilburn et al., 2000). Therefore, we examined HLA-G expression in our model and in contrast to those previous studies observed that extravillous trophoblasts stained strongly for HLA-G throughout the cell columns in both 1.5 and 8% oxygen (Figure 4). In support of our data, more physiologically relevant experiments on outgrowth from explants and isolated first trimester trophoblasts showed no change in the expression of either the membrane-bound or the soluble isoforms of HLA-G between 20 and 2% oxygen (Genbacev et al., 1997; Nagamatsu et al., 2004). Furthermore, our own observations of placental bed biopsies show that HLA-G expression changes only in the proximal extravillous trophoblast columns (Bhalla et al., in press). Once extravillous trophoblasts enter the decidua, HLA-G is not further up-regulated nor is there an apparent difference in the level of HLA-G expression between endovascular trophoblasts which are exposed to arterial oxygen levels and extravillous trophoblasts in the surrounding decidua, which are exposed to lower oxygen levels, suggesting that HLA-G expression is not regulated by oxygen concentration (Bhalla et al., in press). Despite previous reports of oxygen-regulated expression of α₅, the integrin in extravillous trophoblast outgrowths (Genbacev et al., 1997), we found that this molecule, like HLA-G, was expressed throughout the cell columns of all extravillous trophoblast outgrowths, with no difference observed between 1.5 and 8% oxygen conditions (Figure 4).

Therefore, our results suggest that the expression of HLA-G and α₅ integrin is not regulated by oxygen concentration and that neither of these molecules are markers of responses to changing oxygen in trophoblasts.

It is commonly believed that the trophoblast plugs which occlude the maternal spiral arteries gradually disperse from 10 weeks of gestation, allowing maternal blood to flow into the intervillous space inducing a rise in oxygen tension across the villous surface (Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). It was interesting to note that when we examined the ability of explants to produce trophoblast outgrowth via the differential response of explants from the same placenta to 1.5 or 8% oxygen, there was an effect of oxygen concentration only for those placenta of <11 weeks. We had anticipated that there would be a gradual decline in the effect of oxygen concentration over the gestational ages we studied, because the placentae become exposed to increasing oxygen concentrations in vivo. However, our results showed a sharp change in the responsiveness to oxygen, with a clear lack of responsiveness to low oxygen in placenta of 11 weeks and greater. We interpret this result to indicate that there is a shift from a low-oxygen environment around 10 weeks of gestation such that the lack of responsiveness after this time may be either a result of the placenta preparing itself for the entry of maternal arterial blood into the intervillous space or a response to increasing oxygen concentration as the trophoblast plugs dissipate. Our data support the growing body of evidence that the trophoblast plugs dissipate exposing the placenta to maternal blood and increased oxygen concentrations from ~10 weeks of gestation but suggest that there may be a sharp, rather than a gradual, change to arterial-like oxygen concentration in the intervillous space between 10 and 11 weeks (Jauniaux et al., 1992, 2001, 2003; Rodes et al., 1992; Jaffe et al., 1997).

We have analysed our extravillous trophoblast outgrowth frequency data in two separate ways. These two analyses allowed us to investigate the overall effect of gestational age on trophoblast outgrowth, where we showed a progressive
decline in the ability of explants to produce outgrowth with increasing gestational age and, second, a more in-depth analysis using matched explants (differential response) allowed us to demonstrate that oxygen concentration modifies the gestational effect but only for placenta of <11 weeks of gestation with a sharp change in the responsiveness to oxygen between 10 and 11 weeks of gestation. 

In conclusion, we have shown that low oxygen in the culture environment results in a decrease in the frequency and size of extravillous trophoblast outgrowths from villous explants. Furthermore, we have shown a difference in the ability of this tissue to produce extravillous trophoblast outgrowth and respond to changes in oxygen concentration with gestational age. Our results also question whether HLA-G or α1 integrin is regulated by oxygen concentration in the first trimester placenta. The data we have presented clearly show that both gestational age and oxygen play important regulatory roles in extravillous trophoblast outgrowth formation during the first trimester.

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Dear Editors,

We read with interest the paper entitled "Bi-potential Behaviour of Cytotrophoblasts in First Trimester Chorionic Villi" by Baczky et al. published in issue 27 of Placenta.

This paper attempts to address the important question of whether late first trimester placentae contain bipotential cytotrophoblasts that can differentiate into both extravillous trophoblasts and syncytiotrophoblasts. While these workers have observed a number of important phenomena, we are concerned that their central claim, that they have shown the "true bipotential behaviour of a subset of cytotrophoblasts residing on the basal lamina of villi," is not supported by the data presented in the manuscript. We believe their data are open to quite a different interpretation which we discuss here. Baczky et al. described experiments in which they removed the syncytiotrophoblast from placenta of 11-12 weeks of gestation. Under control conditions, the cytotrophoblasts of denuded villi differentiated to reform syncytiotrophoblast. In contrast, denuded villi cultured with FGF-4 failed to regenerate the syncytiotrophoblast. In these FGF-4 treated villi, proliferation of FGF-R2 positive cytotrophoblasts was stimulated, purportedly resulting in the formation of "cytotrophoblast clumps," which exhibited invasive markers indicative of differentiation into extravillous trophoblast. From these results Baczky et al. concluded that FGF-4 redirected differentiation of cytotrophoblasts into an extravillous phenotype rather than to syncytialization and therefore that a population of bipotential cytotrophoblasts exists. While Baczky et al. demonstrated that FGF-4 inhibits syncytialization of villous cytotrophoblasts, their data do not demonstrate that these same cytotrophoblasts were bipotent and induced by FGF-4 to adopt an extravillous phenotype. Baczky et al. examined only placentae of 11-12 weeks of gestation. Such placentae contain many anchoring villi from which extravillous trophoblast columns arise in vivo. In such villi the trophoblast columns form adjacent to deep pockets (or islands) of proliferating villous cytotrophoblasts that have a similar appearance to the "cytotrophoblast clumps" supposedly induced by FGF-4 in the study of Baczky et al. It is unclear to us how Baczky et al. were able to distinguish between pre-existing anchoring villous tips and the "cytotrophoblast clumps" which they claim were formed de novo in denuded villi as a consequence of FGF-4 treatment. Furthermore, if the villous cytotrophoblasts which underlie the denuded syncytiotrophoblast were bipotent then it would be expected that "cytotrophoblast clumps" would form around large areas of the denuded villi at all points where the cultured villi contacted the growth surface as suggested by Aplin et al. [1]. As only one low-power image is presented (figure 3, stained with Ki67), it is very difficult to establish whether this occurred, but from this image the number of trophoblast columns evident does not appear unusual for a placenta of 11 or 12 weeks of gestation. This matter might have been addressed if Baczky et al. have presented a statistical analysis of the numbers of "cytotrophoblast clumps" in FGF-4 treated and control cultures but such analysis is absent in this paper. We have recently published a study in which we suggested that as early as 8 weeks of gestation there are two distinct populations of villous cytotrophoblasts, those found underlying the syncytiotrophoblast and those found in the multilayered pockets adjacent to the extravillous trophoblast columns. These latter cytotrophoblasts we believe are committed to the extravillous pathway and are characterised by their proliferative potential, expression of zvβ5 integrin, and prolonged survival in explant culture [2]. The data of Baczky et al. suggest that these cells are also FGF-R2 positive. Therefore, we believe that the results of Baczky et al. support our contention that villous cytotrophoblasts are not bipotential from 8 weeks of gestation.

In summary, we believe that Baczky et al. did not present evidence showing that FGF-4 can redirect villous cytotrophoblast differentiation of the same cytotrophoblast population down either the extravillous or syncytiotrophoblast pathway. We do not believe the conclusion that a subset of cytotrophoblasts is bipotent as an accurate summation of the data in this paper as it is possible that FGF-4 may merely have different actions on two cell subpopulations — acting to stimulate proliferation of extravillous trophoblast progenitors, while inhibiting the fusion of monolayer villous cytotrophoblasts into syncytiotrophoblast.
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Yours sincerely

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The Effects of Oxygen Concentration and Gestational Age on Extravillous Trophoblast Outgrowth from First Trimester Villous Explants.

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In the first trimester of human pregnancy, extravillous trophoblasts from anchoring placental villi invade the decidua and transform the maternal spiral arteries. Extravillous trophoblasts temporarily occlude the spiral arteries preventing maternal blood flow and creating a low oxygen environment, which is believed to play an important role in the regulation of extravillous trophoblast outgrowth from villi during the first trimester. Therefore, the purpose of this work was to investigate and quantify the effects of gestational age, oxygen concentration, and the interaction between these factors, on extravillous trophoblast outgrowth from first trimester villi. A quantitative two-dimensional villous explant model was used to measure the frequency and area of extravillous trophoblast outgrowths from 3963 explants grown in 1.5% and 8% oxygen conditions. Gestational age affected outgrowth independently of oxygen concentration, with the percentage of explants producing outgrowth declining as gestational age increased from 8 to 12 weeks. Culture in 1.5% oxygen significantly reduced the frequency and area of extravillous trophoblast outgrowths in comparison to culture in 8% oxygen. The decreased area of extravillous trophoblast outgrowth in 1.5% oxygen can be predominantly attributed to a decrease in the area of individual extravillous trophoblasts, however factors influencing trophoblast proliferation and differentiation may also contribute. Gestation also influenced the response of explants to low oxygen conditions with a significant differential response to oxygen concentration in placentae under 11 weeks of gestation whereas, in villi from placentae of 11 or 12 weeks there was no differential response to oxygen concentration. Thus, in the first trimester oxygen and gestational age both play an important regulatory role in extravillous trophoblast outgrowth.

Annual Meeting of the Society for Reproductive Biology, Brisbane, Australia, August 2006.
Isolation of ‘Extravillous Trophoblast Progenitors’ from First Trimester Villi

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Objective: A single pool of villous cytotrophoblast are widely believed to be precursors of both syncytiotrophoblast and extravillous trophoblast (EVT) in the first trimester of human pregnancy. We hypothesised that from at least 8 weeks of gestation syncytiotrophoblast and EVTs arise from different villous cytotrophoblast progenitor populations. We have previously shown that the progenitors of EVTs are localised to the, the multilayered pockets of villous cytotrophoblasts in the tips of anchoring villi and that these cells can survive for prolonged periods in explant culture, whereas the majority of villous cytotrophoblasts die within a week in explant culture. This study was conducted to determine whether EVT progenitors could be isolated based on their extended survival in explant culture. Methods: First trimester villous explants were cultured for 1 week. Cytotrophoblasts were then isolated from the explants following sequential digestion with trypsin. The cells from each digest were cultured on a thin layer of Matrigel. Non-adherent cells were removed by washing. Cell viability was confirmed by trypan blue exclusion and the cells were phenotyped by immunohistochemistry. Results: Viable trophoblasts (>95% purity) were obtained from the 3rd and 4th trypsin digests which formed into clusters of proliferating cells, as confirmed by Ki67 expression. The proliferating trophoblasts expressed cytokeratin and αvβ6 integrin, but did not express the ECM protein tenascin. Conclusions: The method of explant culture followed by trypsin digestion has allowed us to isolate a pool of proliferating trophoblast that express markers consistent with the cells we propose are EVT progenitors. This method will allow further characterisation of these cells.

The Effect of Hypoxia on Extravillous Trophoblast Outgrowth in a Quantitative Two-Dimensional Explant Model

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In the first trimester of human pregnancy the presence of trophoblast plugs in the maternal spiral arteries occludes the flow of maternal blood to the intervillous space and creates a hypoxic environment, which is believed to play an important role in the regulation of placental development and the success of pregnancy. The aim of this research was to determine the effect of hypoxia on the production of extravillous trophoblast outgrowth in vitro with respect to the size of the outgrowth produced, and the expression of molecules associated with trophoblast differentiation into an invasive phenotype. **Methods:** First trimester villous explants from placentae of 8 to 12 weeks of gestation were cultured in a two-dimensional Matrigel model in atmospheres of either 1.5% or 8% oxygen. Extravillous trophoblast outgrowth was quantified by digital image analysis. Extravillous trophoblasts were stained with an antibody to HLA-G. **Results:** A significant decrease in the number of explants producing extravillous trophoblast outgrowth was observed when explants were cultured under 1.5% oxygen (12.9%) compared to 8% oxygen (17.46%, p=0.001). Furthermore, extravillous trophoblast outgrowth produced by explants cultured under 1.5% oxygen (0.94mm², n=208) was significantly smaller than from explants cultured under 8% oxygen (1.20mm², n=269, p=0.04). But there was no difference in the expression of HLA-G by the extravillous trophoblast outgrowth under 1.5% or 8% oxygen conditions. **Conclusions:** In this model oxygen appears to regulate the formation of extravillous trophoblast outgrowth, but not extravillous trophoblast HLA-G expression.

10th Meeting of the International Federation of Placenta Associations, Monterey, California, USA. Published in Placenta 25(8-9): A17, 2004.
Viability of First Trimester Placental Villous Explants In Vitro

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The aim of this study was to investigate the relationship between the viability of first trimester chorionic villous tissue and the formation of extravillous trophoblast outgrowth. **Methods:** In order to examine cellular viability, first trimester villous explants were stained with chloromethylfluorescein diacetate (CMFDA) and ethidium bromide immediately after collection, or after culture on a thin layer of Matrigel for 24, 48 or 96 hours. To determine the effect of prolonged culture on extravillous trophoblast outgrowth villous explants were cultured for 7 days then passaged twice into fresh Matrigel-coated culture plates. **Results:** Degeneration of the syncytiotrophoblast was observed within 4 hours of commencing the cultures. From 48 hours of culture the syncytiotrophoblast layer had regenerated, whereas the vast majority of the underlying cytotrophoblast layer and cells of the mesenchymal core were no longer viable. However, the multilayered cytotrophoblasts located in villous tips behind the extravillous trophoblast columns remained viable. Production of extravillous trophoblast outgrowth was able to continue for up to three weeks in culture. **Conclusions:** The continued expansion of extravillous trophoblast outgrowth for three weeks despite the widespread death of the majority of the villous cytotrophoblasts and cells of the mesenchymal core during the early stages of culture suggests that extravillous trophoblast outgrowth is not dependent on either of these cell types. Therefore it appears that the only placental factor required for the production of extravillous trophoblast outgrowth was the multilayered villous cytotrophoblasts from which the columns originate.

10th Meeting of the International Federation of Placenta Associations, Monterey, California, USA. Published in Placenta 25(8-9): A17, 2004.
Life and Death in a First Trimester Villous Explant Model

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In first trimester placental anchoring villi, cytotrophoblasts break through the syncytiotrophoblast layer and form columns of extravillous trophoblasts. Extravillous trophoblasts physically attach the placenta to the decidual stroma and modify the spiral arterioles in the process of placentation. The factors causing differentiation of cytotrophoblasts into extravillous trophoblasts rather than syncytium in anchoring villi are unknown. Therefore, the aim of this study was to investigate the relationship between the viability of first trimester chorionic villous tissue and the formation of extravillous trophoblast outgrowth. This study was undertaken using a first trimester villous explant culture model from which extravillous trophoblast outgrowth can be quantified. Dual staining of villous explants with chloromethylfluorescin diacetate (CMFDA) and ethidium bromide revealed degeneration of the syncytiotrophoblast within 4 hours of commencing the cultures. However, by 48 hours of culture the syncytiotrophoblast layer had regenerated, whereas the vast majority of the underlying cytotrophoblast layer and cells of the mesenchymal core were no longer viable. Despite this, the production of extravillous trophoblast outgrowth was able to continue for up to three weeks in culture. Therefore, the only factor required for the production of extravillous trophoblast outgrowth was viable cytotrophoblasts in the villous tip from which the outgrowth was produced.

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