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Cytotrophoblast Differentiation in the First Trimester of Human Pregnancy

Joanna James

A thesis submitted in partial fulfillment of the requirements for the degree
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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.

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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 EVT's 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 chloromethylfluorescein 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 $\alpha v\beta 6$ 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 $\alpha 1$ 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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List of Common Abbreviations

bHLH	basic helix loop helix
CMFDA	chloromethylfluorescein diacetate
CDK	cyclin dependent kinase
CSF-1	colony stimulating factor-1
CVS	chorionic villous sampling
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EtBr	ethidium bromide
EVT	extravillous trophoblast
FGF-4	fibroblast growth factor-4
FGFR-2	fibroblast growth factor receptor-2
GM-CSF	granulocyte macrophage – colony stimulating factor
hCG	human chorionic gonadotrophin
HIF	hypoxia inducible factor
HLA	human leukocyte antigen
IGF	insulin growth factor
IGFBP	insulin growth factor binding protein
IVS	intervillous space
IL	interleukin
LIF	leukaemia inhibitory factor
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline solution
PCNA	proliferating cell nuclear antigen
PI3K	phosphatidylinositol 3 kinase
PlGF	placental growth factor
PS	phosphatidyl serine
pVHL	von Hippel-Lindau tumour suppressor protein
TGF	transforming growth factor
TGFR	transforming growth factor receptor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumour necrosis factor
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor