Insulin-Like Growth Factor –II and its Role in Blastocyst Development, Implantation and Placentation

Kirsty Gay Pringle

Research Centre for Reproductive Health

Discipline of Obstetrics & Gynaecology

The University of Adelaide, Adelaide

Australia

Thesis submitted to the University of Adelaide in fulfillment of the requirements for admission to the degree Doctor of Philosophy

December 2007

TABLE OF CONTENTS

ABSTRACT

Impaired implantation and placental development have been implicated in several disorders of pregnancy such as unexplained miscarriage, preeclampsia, and intrauterine growth retardation. Insulin-Like Growth Factor (IGF)-II has previously been shown to promote blastocyst development and placental growth and function. We were interested in how IGF-II interacts with other factors throughout blastocyst development, implantation and placentation in the mouse to improve pregnancy outcome.

In vitro embryo culture increases the risk of pregnancy complications associated with poor placentation. Recent research has focussed on optimising the culture conditions to more resemble that of the *in vivo* environment. IGF-II, Urokinase Plasminogen Activator (uPA) and Plasminogen individually have all been shown to be important for embryo development. However, it is likely that a combination of factors is required to counteract the negative effects of *in vitro* culture. Here we show that IGF-II, uPA and Plasminogen, in combination, significantly improve mouse blastocyst hatching rates and implantation rates on day 8 and doubles the number of mothers that are pregnant after embryo transfer.

Following implantation, IGF-II is suggested to play a role in promoting placental development and function. We demonstrate that IGF-II is co-localised with both IGF receptors throughout early pregnancy in trophoblasts and in the developing blood vessels and adjacent stromal cells in the mesometrial decidua. This suggests that IGF-II may play a role in both decidual angiogenesis and placentation. We suggest that perhaps murine trophoblasts secrete molecules such as IGF-II to promote

angiogenesis in the decidua early in pregnancy to compensate for their shallow invasion and allow for adequate trophoblast remodelling later in pregnancy.

The first trimester human placenta experiences a low oxygen environment. The Hypoxia-Inducible Factors (HIFs) mediate the response to low oxygen, inducing genes such as IGF-II. Currently, the role of oxygen in mouse placentation, the mechanisms by which HIFs promote placentation or their interaction with IGF-II in the placenta is unknown. Here, we demonstrate that the early mouse implantation site is exposed to low oxygen levels similar to those seen in humans and expresses HIF-1α protein. We were interested then in the interaction between IGF-II, oxygen and HIFs in trophoblasts *in vitro*. Prolonged exposure to low oxygen reduced trophoblast outgrowth, and increased *Tpbp* mRNA levels, suggesting commitment to the spongiotrophoblast lineage. Interestingly, we found that antisense (as) *Hif-1α* may mediate the response to prolonged hypoxia in murine trophoblasts. Importantly, *Hif-1α* and *Hif-2α* were differentially regulated by oxygen and IGF-II in cultured trophoblast cells suggesting a novel interaction between IGF-II and oxygen.

In conclusion, it appears that IGF-II is a central growth factor which interacts with other molecules to regulate a wide variety of process in early pregnancy to promote blastocyst development, implantation and placentation. The results outlined in this thesis demonstrate a novel interaction between IGF-II, uPA and Plasminogen in promoting blastocyst development and implantation which may be used to improve pregnancy outcome following ART. In addition, we have also identified a novel interaction between IGF-II, oxygen and the HIF system which may regulate trophoblast function. This has important implications not only for placental research, but also for cancer research.

DECLARATION

This work is original and has not been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge, this thesis does not contain material previously written or published by another, except where due reference in the text has been given.

I give consent to the University of Adelaide to make this thesis available for loan and photocopying after it has been accepted for the degree.

Kirsty Gay Pringle

December 2007

ACKNOWLEDGEMENTS

First and foremost I would like to extend an enormous thankyou to my supervisor Associate Professor Claire Roberts. I can't believe it has been 5 years since we first met and you told me all about this wonderful organ, the placenta. It has been an honour to work with someone so dedicated and so passionate about science, you have been an inspiration. Thank you for encouraging me to grow both as a scientist, and as an individual. I would also like to thankyou for believing in me, for pushing me to be the best that I could be and for staying optimistic when I couldn't see the light at the end of the tunnel. Most importantly, I would like to say how grateful I am for your understanding, for letting me cry on your shoulder (one too may times I think) and for being a friend. It has been both a pleasure and an honour.

Secondly, I would like to say a huge thank you to my co-supervisor Dr Karen Kind for your insight and advice. Your input into this project has always been highly regarded and I really appreciated all of the feedback you have given me over the years. It was nice to know that you were always there when I needed you. In particular I would like to thankyou for letting me pick your brains so often, whether it was about real time PCR or what the heck my results meant!

In addition I would like to acknowledge all of the past and present members of the Placental Development Group that I have had the pleasure of working with. I have gained some wonderful friends and it wouldn't have been the same without you. I would also like to acknowledge Amanda Seferruzzi-Perri, Rachael Nowak, Jamie Zhang, Steven Thompson and Gary Heinemann for their technical assistance with the embryo transfer experiments.

To all of the wonderful staff and students in the Research Centre for Reproductive Health at the University of Adelaide, thank you for making my time in the department so enjoyable. In particular I would like to thank Kylie Dunning, Rachael Nowak and Amanda Sferruzzi-Perri, my PhD buddies, for your understanding, love and support. There is no way I could have made it to the end without you girls!

I would like to acknowledge the financial support of the University of Adelaide, the Faculty of Health Sciences, the Research Centre for Reproductive Health (RCRH) and the Network in Genes and Environment in Development (NGED) for my postgraduate scholarship and for national and international travel grants. In addition, the work outlined in Chapter 2 was financially supported by Medicult.

Lastly, I would like to thank all the other people in my life that I couldn't live without. To my family, Mum & Phil, Dad & Angela, Jay and Joel, I would like to thankyou from the bottom of heart for your unconditional love and support. In particular I would like to extend an enormous thanks to my mum for being there through everything. Thankyou for knowing when to push me and when to let things go and for picking me up when I didn't think I could go on. Thank you to Sadie Peck for always being a wonderful friend and to the Kahl family, for letting me into their home and their hearts. Last but not least, I would like to thank Richard for loving me, encouraging me and supporting me. I can't begin to describe how grateful I am to have you in my life, I love you very much.

PUBLICATIONS ARISING FROM THIS THESIS

1. **Pringle KG**, Kind KL, Thompson JG, Roberts CT. *Complex Interactions Between Hypoxia Inducible Factors, Insulin-like Growth Factor-II and Oxygen in Early Murine Trophoblasts.* Placenta. 2007; Nov-Dec; 28(11-12): 1147-57.

2. **Pringle KG**, Roberts CT. *New light on Early Post-Implantation Pregnancy in the Mouse: Roles for Insulin-Like Growth Factor-II (IGF-II)?* Placenta. 2007 Apr; 28(4): 286-97.

3. **Pringle KG**, Kind KL, Thompson JG, Roberts CT. Control of Placental Development by Oxygen and Hypoxia Inducible Factors. In preparation.

4. **Pringle KG,** Kind KL, Thompson JG, Roberts CT. IGF-II, in combination with uPA and Plasminogen, improves mouse blastocyst development and implantation. In preparation.

ADDITIONAL PUBLICATIONS

5. Sferruzzi-Perri AN, Owens JA, **Pringle KG**, Robinson JS, Roberts CT. *Maternal Insulin-Like Growth Factors-I and -II Act via Different Pathways to Promote Fetal Growth.* Endocrinology. 2006 Jul; 147(7): 3344-55.

ABSTRACTS ARISING FROM THIS THESIS

2007

1. **Pringle KG**, Kind KL, Thompson JG and Roberts CT. *Effect of IGF-II, uPA and Plasminogen in Combination on Blastocyst Development.* Society for Reproductive Biology Annual Scientific Meeting, Christchurch, New Zealand.
2. ***Pringle KG,*** Kind KL, Thompson JG and Roberts CT. *Oxygen, IGF-II and their Interactions in Early Murine Trophoblasts.* 13th meeting of the International Federation of Placenta Associations (IFPA), Kingston, Ontario, Canada.
3. **Pringle KG**, Kind KL, Thompson JG, Roberts CT. *Oxygen, IGF-II and their Interactions in Murine Trophoblasts.* Network in Genes and Environment in Development (NGED) Forum. Palm Cove, Queensland, Australia.

2006

4. **Pringle KG**, Kind KL, Thompson JG and Roberts CT. *Oxygen, Insulin-Like Growth Factor-II (IGF-II) and Their Interactions in Murine Trophoblasts in Vitro.* Abstract 208. Society for Reproductive Biology Annual Scientific Meeting, Gold Coast, Queensland, Australia.
5. Sferruzzi-Perri AN, Owens JA, **Pringle KG**, Robinson JS, Roberts CT. *Maternal Insulin-Like Growth Factor-I and –II Act Via Different Pathways to Increase Fetal Growth Near Term.* Abstract 221. Society for Reproductive Biology Annual Scientific Meeting, Gold Coast, Queensland, Australia.
6. **Pringle KG**, Kind KL, Thompson JG and Roberts CT. *Interactions between the Hypoxia Inducible Factors and Insulin-Like Growth Factor-II (IGFII) in Cultured Murine Trophoblasts.* Australian Society for Medical Research (ASMR) SA Division Annual Scientific Meeting, Adelaide.

7. Sferruzzi-Perri AN, Owens JA, **Pringle KG**, Robinson JS, Roberts CT. *Exogenous insulin-like growth factors in early pregnancy act via different mechanisms to promote fetal growth.* 11th meeting of the International Federation of Placenta Associations (IFPA), Glasgow, Scotland.
8. **Pringle KG** and Roberts CT. *IGF-II and IGF2R expression in early murine implantation sites: a role in both placentation and decidual angiogenesis?* 32nd Annual Meeting of the Fetal and Neonatal Physiological Society, Jeffrey Robinson Symposium, Glenelg, South Australia, Australia.
9. Sferruzzi-Perri AN, Owens JA, **Pringle KG**, Robinson JS, Roberts CT. *Maternal IGF-II treatment in Early pregnancy improves placental structural and functional development and fetal growth near term*. 32nd Annual Meeting of the Fetal and Neonatal Physiological Society, Jeffrey Robinson Symposium, Glenelg, South Australia, Australia.
10. **Pringle KG** & Roberts CT. *Localisation of Insulin-Like Growth Factor-II (IGF-II) and its Receptor in Early Murine Pregnancy: A Role in Placentation and Angiogenesis in the Decidua?* Abstract 210. Society for Reproductive Biology Annual Scientific Meeting, Perth, Western Australia, Australia.
11. **Pringle KG**, Kind KL, Thompson JG and Roberts CT. *Effect of oxygen and insulin-like growth factor-II on ectoplacental cone outgrowth and trophoblast gene expression.* Abstract A162. 9th Annual Conference of the Perinatal Society of Australia and New Zealand (PSANZ), Adelaide, South Australia, Australia.
12. **Pringle KG**, Kind KL, Thompson JG & Roberts CT. *Role of oxygen and IGF-II on early trophoblast outgrowth and gene expression in the mouse.* Abstract A1. 19th National Workshop on Fetal and Neonatal Physiology, Adelaide.

13. **Pringle KG** & Roberts CT. *Localisation of IGF-II and IGF2R in Murine Pregnancy: A Role in Both Placentation and Decidual Angiogenesis?* Abstract 02. Australian Society for Medical Research (ASMR) SA Division Annual Scientific Meeting, Adelaide.
14. Sferruzzi-Perri AN, Owens JA, **Pringle KG**, Robinson JS and Roberts CT. *Maternal IGF-II treatment in early pregnancy impacts on placental structure to promote fetal growth near term.* Abstract R2. Australian Society for Medical Research (ASMR) SA Division Annual Scientific Meeting, Adelaide.

2004

15. **Pringle KG**, Kind KL and Roberts CT*. Oxygen, Insulin-Like Growth Factor-II (IGF-II) and Hypoxia Inducible Factor-1 alpha (HIF-1α) in the murine placenta.* Abstract A134. 8th Annual Conference of the Perinatal Society of Australia and New Zealand (PSANZ), Sydney.
16. **Pringle KG**, Kind KL and Roberts CT. *Localisation of hypoxia, Insulin-Like Growth Factor-II (IGF-II) and Hypoxia Inducible Factors (HIFs) in early murine implantation sites.* Abstract A10. 18th National Workshop on Fetal and Neonatal Physiology.
17. **Pringle KG**, Kind KL and Roberts CT. *Oxygen, IGF-II and HIF-1α in early murine placentation.* Abstract O4. Australian Society for Medical Research (ASMR) SA Division Annual Scientific Meeting, Adelaide.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

asHIF-1α	antisense hypoxia inducible factor -1 alpha
ALS	acid labile subunit
Ang-II	angiotensin - II
ANOVA	analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator
ART	assisted reproductive technology
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
CAD	C-terminal activation domain
CBP	CAP binding protein
cDNA	complementary DNA
CI-M6PR	cation-independent mannose-6 phosphate receptor
CTB	cytotrophoblast
DAB	diaminobenzadine
DNA	deoxyribonucleic acid
eCG	equine chorionic gonadotrophin
ECM	extracellular matrix
EGF	epidermal growth factor
EPAS-1	endothelial PAS domain protein 1
EPC	ectoplacental cone
EPO	erythropoietin
EVT	extravillous cytotrophoblast
ExE	extraembryonic ectoderm

FIH	factor inhibiting HIF
GLUT-1	glucose transporter – 1
H&E	haematoxylin and eosin
HBSS	hank's balanced salt solution
hCG	human chorionic gonadotrophin
HIF	hypoxia inducible factor
HPH	HIF prolyl hydroxylase
HRE	hypoxia response element
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IGF1R	type 1 insulin-like growth factor receptor
IGF2R	type 2 insulin-like growth factor receptor
IGFBP	insulin-like growth factor binding protein
IGFBPrP	insulin-like growth factor binding protein related protein
ICM	inner cell mass
IL-1β	interleukin -1 beta
i.p	intraperitoneal
IPAS	inhibitory PAS domain protein
IR	insulin receptor
IUGR	intrauterine growth restriction
IVF	*in vitro* fertilisation
IVS	intervillous space
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
M6PR	mannose-6 phosphate receptor
MAPK	mitogen activated protein kinase

MLAp	mesometrial lymphoid aggregate of pregnancy
MMP	matrix metalloproteinase
ODD	oxygen dependent degradation
NAD	N-terminal activation domain
NTC	non-template control
pAb	polyclonal antibody
PBS	phosphate buffered saline
PDZ	primary decidual zone
PHD	prolyl-4 hydroxylase
P_4	progesterone
PAI	plasminogen activator inhibitor
PAS	Per-Arnt-Sim
PC4	proprotein convertase 4
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PDGF	platelet - derived growth factor
PGE_2	prostaglandin E_2
PH	proline-4 hydroxylase related protein
PHD	prolyl -4 hydroxylase
PI3K	phosphatidylinositol-3 kinase
PL-1	placental lactogen-1
PLC	phospholipase C
Plg	plasminogen gene
RNA	ribonucleic acid
RT	reverse transcription
SEM	standard error of the mean

SGA	small for gestational age
STB	syncytiotrophoblast
TE	trophectoderm
TGC	trophoblast giant cell
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau
VDU2	VHL-interacting deubiquitinating enzyme 2

Chapter 1
Introduction

1.1 Introduction

Implantation and placental development are critical to establish physical connections between the embryo and the mother for the embryo's survival. The placenta ensures an adequate nutrient supply from the mother to the fetus and creates an appropriate hormonal environment necessary to maintain pregnancy. The placenta also acts to protect the fetus from the maternal immune system, preventing rejection (Cross, Werb et al. 1994; Rossant and Cross 2001; Georgiades, Ferguson-Smith et al. 2002).

The human placenta is analogous to a locally invasive tumour (Lala and Graham 1990; Foidart, Hustin et al. 1992). The trophoblasts of the first trimester placenta share some phenotypic similarities with cancerous cells e.g. rapid proliferation and the ability to invade neighbouring tissue (Yagel, Parhar et al. 1988). In some cases, invasion of the uterus continues indefinitely, leading to choriocarcinoma (Graham, Connelly et al. 1994). Fortunately, during normal pregnancy, trophoblast invasion of the uterus is under strict control (Lala and Graham 1990). Conversely, impaired trophoblast invasion has been implicated in several complications of pregnancy such as unexplained miscarriage, gestational diabetes, preeclampsia, and intrauterine growth restriction (IUGR) (Georgiades, Ferguson-Smith et al. 2002). In preeclampsia and some miscarriages, for example, invasion of the spiral arterioles and the maternal decidual stroma is shallow, resulting in poor maternal blood flow to the placenta (Khong, Liddell et al. 1987; Rinkenberger, Cross et al. 1997; Zhou, Genbacev et al. 1998). Interestingly, women undergoing assisted reproductive technologies (ART) are at an increased risk of developing pregnancy complications associated with poor placentation such as preeclampsia, placenta previa, placental abruption and gestational diabetes (Shevell, Malone et al. 2005; Reddy, Wapner et

al. 2007). ART has also been associated with an increased risk of preterm delivery, low birth weight, small for gestational age (SGA) and perinatal mortality (Doyle, Beral et al. 1992; Shevell, Malone et al. 2005; Poikkeus, Gissler et al. 2007; Reddy, Wapner et al. 2007).

Implantation of the blastocyst and formation of the placenta involves the complex regulation of trophoblast proliferation, differentiation and invasion. Growth factors (insulin-like growth factor (IGF)-I and –II, epidermal growth factor (EGF), transforming growth factors (TGFs) and vascular endothelial growth factor (VEGF)), cell adhesion molecules (integrins, cadherins), extracellular matrix (ECM) components and their regulators (collagen, vitronectin, matrix metalloproteinases (MMPs), plasminogen activator inhibitors (PAI), plasminogen), oxygen and its mediators (hypoxia inducible factors), cytokines and hormones, all influence trophoblast function (Morrish, Dakour et al. 1998; Lala and Chakraborty 2003). There is a significant body of evidence to suggest that IGF-II is a principal regulator of trophoblast function, therefore this is the focus of our studies. In particular, this thesis will examine the interactions between IGF-II and other important regulators including oxygen, HIFs and mediators of ECM degradation and cellular migration (the urokinase plasminogen activator system), in modulating blastocyst implantation and trophoblast migration and differentiation.

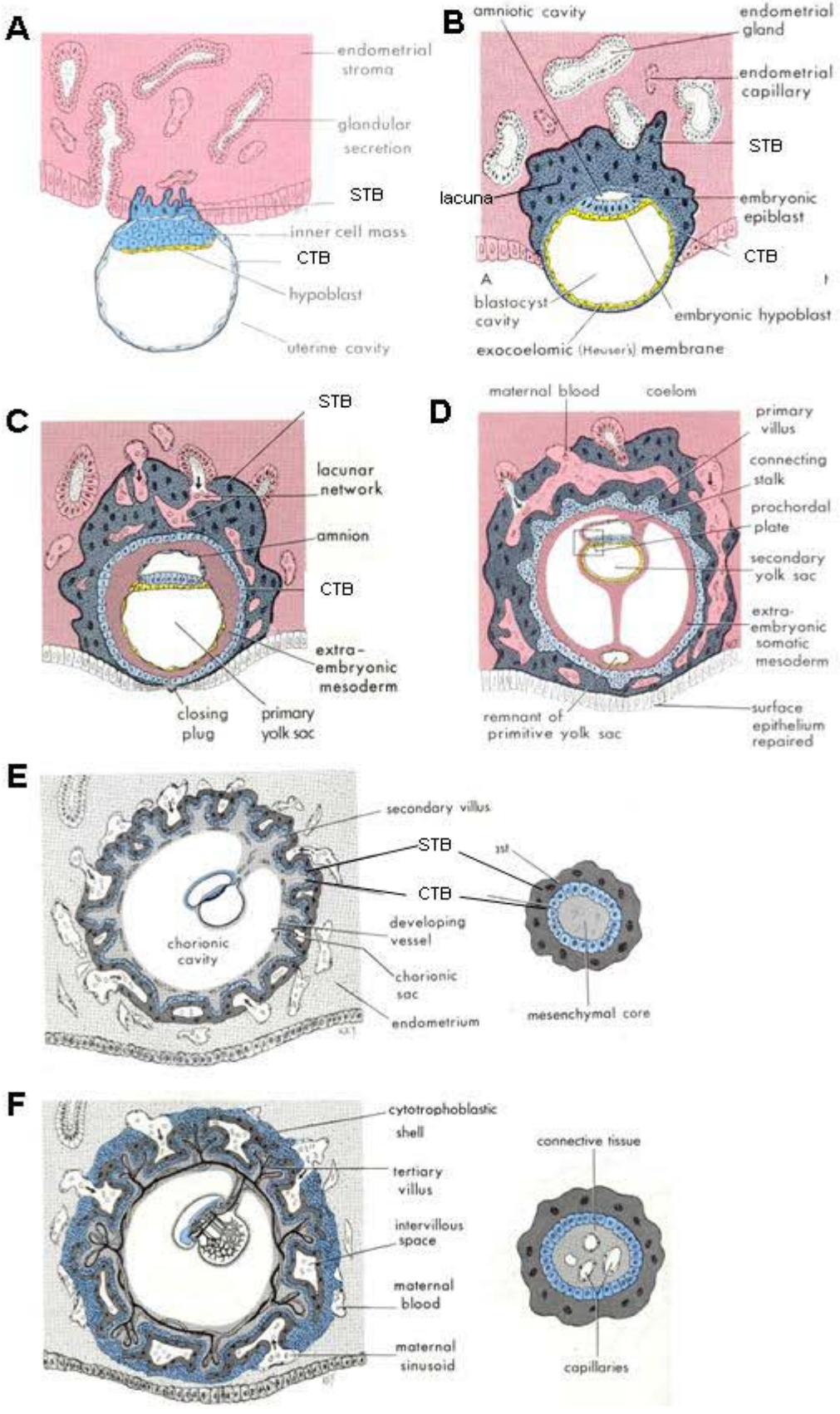
1.2 Implantation and Placentation

1.2.1 Implantation and Placentation in Humans

After blastocyst hatching from the zona pellucida the trophectoderm becomes attached to the maternal uterine epithelium on the posterior uterine wall. The embryo then penetrates the epithelium becoming embedded in the maternal stroma. Upon implantation, the trophoblasts differentiate into 2 layers: 1) an outer

syncytiotrophoblast (STB) or syncytium, and 2) an inner cytotrophoblast (CTB) which gives rise to cells that fuse to form more STB (Moore 1988) (Figure 1-1). By the end of the second week, continued proliferation of the CTBs produces primary chorionic villi, which evaginate the STB. These villi begin to branch, forming secondary chorionic villi which have a core of mesenchyme (Figure 1-1). The mesenchymal cells soon differentiate to form capillaries and vessels which are connected with the embryo, at which stage the villi are called tertiary chorionic villi (Moore 1988). Two types of villi exist (Figure 1-2). In floating villi, CTBs fuse to form STB that covers the chorionic villi and interacts directly with maternal blood in the intervillous space (IVS). Floating villi comprise the fetal compartment involved in placental exchange, they are bathed by maternal blood and perform gas and nutrient exchange functions for the developing embryo (Moore 1988; Damsky, Fitzgerald et al. 1992; Zhou, Genbacev et al. 1998). The CTBs in anchoring villi have an additional function. As well as giving rise to the multinucleated syncytium, some CTBs breach the syncytiotrophoblastic layer forming columns of extravillous cytotrophoblasts (EVTs) (Damsky, Fitzgerald et al. 1992; Bischof, Meisser et al. 2000), which join to form a cytotrophoblastic shell (Figure 1-2). These EVTs are able to invade into the endometrium and the first third of the myometrium (interstitial invasion), as well as the uterine spiral arterioles (endovascular invasion) (Pijnenborg, Robertson et al. 1981; Moore 1988; Cross, Werb et al. 1994; Genbacev, Joslin et al. 1996; Genbacev, Zhou et al. 1997; Zhou, Genbacev et al. 1998). Fetal CTBs replace the maternal endothelium, resulting in a dramatic increase in the diameter of the spiral arterioles, allowing blood flow to the placenta to keep pace with the growing fetus (Zhou, Genbacev et al. 1998).

Figure 1-1 Schematic representation of early human implantation and placentation
On days 5 and 6 of gestation, the blastocyst attaches to the uterine epithelium and embeds into the uterine stroma (**A**). Upon implantation the trophoblasts differentiate into two layers, an outer syncytiotrophoblast (STB) and an inner cytotrophoblast (CTB) cell layer. Lacunae begin to appear within the STB (**B**). On day 10, the embryo completely implants into the endometrium and the lacunae fuse to form lacunar networks, the precursor to the intervillous spaces (**C**). Primary chorionic villi form from the proliferation of CTB beneath the STB between days 11 and 13 (**D**). By days 15 to 16 of pregnancy, these villi begin to branch, forming secondary chorionic villi (depicted in cross-section) which have a core of mesenchyme (**E**). These villous mesenchymal cells differentiate into fetal capillaries from day 21 of gestation, they connect to the embryo and form tertiary chorionic villi (seen in cross section) (**F**). Modified from (Moore 1974).


A
endometrial stroma
glandular secretion
STB
inner cell mass
CTB
hypoblast
uterine cavity
B
amniotic cavity
endometrial gland
endometrial capillary
STB
lacuna
embryonic epiblast
CTB
blastocyst cavity
embryonic hypoblast
exocoelomic (Heuser's) membrane
C
STB
lacunar network
amnion
CTB
extra-embryonic mesoderm
closing plug
primary yolk sac
D
maternal blood
coelom
primary villus
connecting stalk
prochordal plate
secondary yolk sac
extra-embryonic somatic mesoderm
remnant of primitive yolk sac
surface epithelium repaired
E
secondary villus
STB
CTB
developing vessel
chorionic cavity
chorionic sac
endometrium
mesenchymal core
F
cytotrophoblastic shell
tertiary villus
intervillous space
maternal blood
maternal sinusoid
connective tissue
capillaries

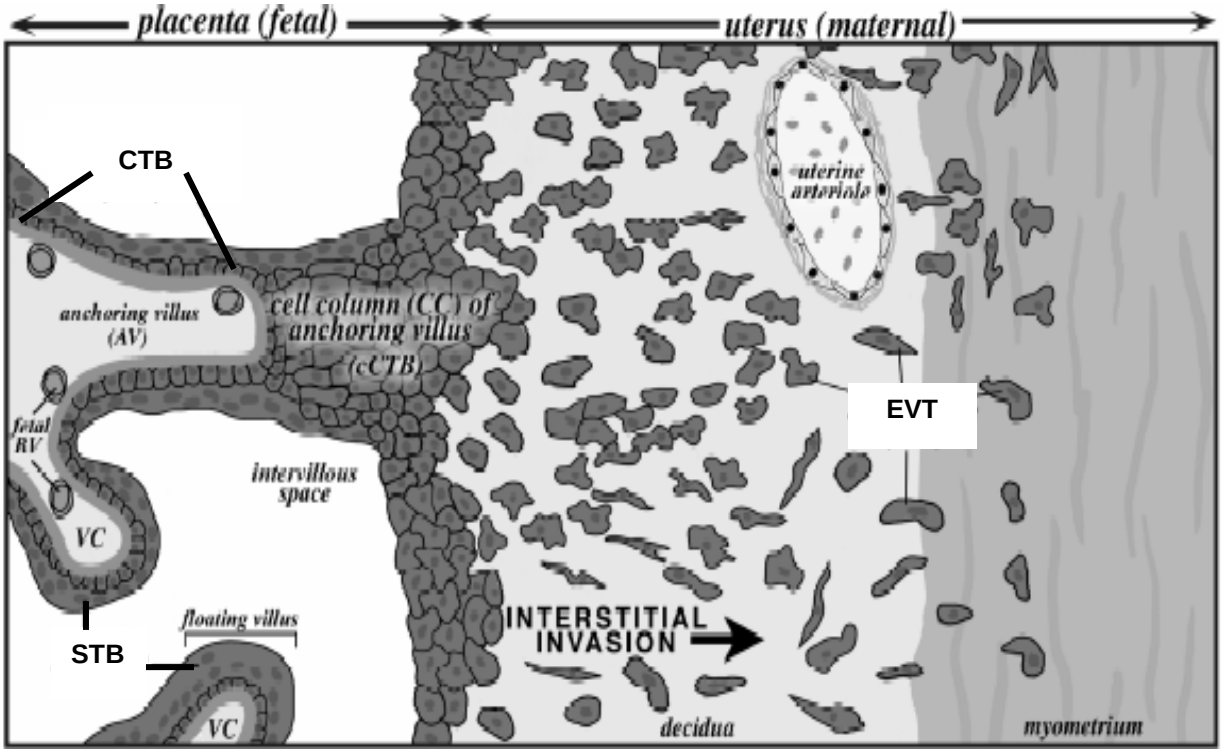


Figure 1-2 Schematic representation of the materno-fetal interface in early human pregnancy
There are two types of villi in the human placenta. Floating villi, which are bathed in maternal blood in the intervillous space and perform gas and nutrient exchange, and anchoring villi that have an additional function. CTBs from anchoring villi proliferate and breach the overlying syncytium to form cell columns which join to form a cytotrophoblastic shell. Column CTBs then differentiate into invasive extravillous cytotrophoblasts (EVTs), which invade into the maternal interstitium and its vasculature. Modified from (Genbacev, Krtolica et al. 2001).

1.2.2 Implantation and Placentation in the Mouse

In mice, the blastocyst attaches to the epithelium in a uterine crypt on day 4.5 of pregnancy via the mural trophectoderm, that which is opposite the inner cell mass (ICM), initiating implantation (Rinkenberger, Cross et al. 1997). By day 5.5 the peripheral, flattened trophoblast cells of the embryo are transformed into trophoblast giant cells (TGCs), which phagocytose the adjacent apoptotic epithelium and gain access to the uterine stroma (Theiler 1972; Blankenship and Given 1992). On day 7.5 in mice, and after implantation, the trophectoderm proliferates to form the ectoplacental cone (EPC) and extraembryonic ectoderm (ExE) (Theiler 1972; Rinkenberger, Cross et al. 1997) (Figure 1-3). The trophoblast cells in the outer regions of the EPC differentiate into secondary trophoblast giant cells, and form the interface between the placenta and the maternal decidua throughout most of pregnancy (Cross, Werb et al. 1994). Adjacent to the chorionic plate is the labyrinthine layer which forms from the fusion of the embryo derived allantois with the chorion (Figure 1-3). The labyrinth consists of fetal vessels and maternal blood spaces, separated by three layers of trophoblast (Enders and Blankenship 1999). Further toward the maternal decidua is the junctional zone, derived from the EPC, which contains no fetal blood vessels but only maternal blood spaces and spongiotrophoblasts. Many of the trophoblast cells in this region differentiate to form trophoblast glycogen cells (Pijnenborg, Robertson et al. 1981). Bordering the maternal side of the junctional zone is the zone of giant cells (Theiler 1972).

Like humans, there are two types of invasion in murine placentation, both interstitial and endovascular (Figure 1-3). However, in the mouse these two types of invasion are spatio-temporally disctinct and are mediated by different trophoblast lineages (Adamson, Lu et al. 2002). Firstly, the TGCs invade endovascularly. However, unlike

humans the vascular remodelling that results in dilation of the spiral arterioles is believed to occur independently of the invading TGCs, although an indirect role has not been ruled out (Adamson, Lu et al. 2002). Later in pregnancy the trophoblast glycogen cells invade interstitially into the maternal decidua, greatly increasing the surface area of the placenta for exchange (Adamson, Lu et al. 2002).

NOTE: This figure is included on page 9 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-3 Placental development in the mouse. By day 3.5 the mouse blastocyst has been formed (**A**). On day 5.5 the blastocyst attaches to the uterine epithelium in a uterine crypt and the cells of the trophectoderm (TE) layer are transformed into trophoblast giant cells (TGCs). Upon implantation the trophectoderm closest to the inner cell mass proliferates, giving rise to the ectoplacental cone (EPC) (**B**). On day 8.5 the embryo derived allantois attaches to the chorion (**C**). Following chorioallantoic fusion and branching of the fetal vessels, villi are formed (**D**). On day 9.5 the three layers of the placenta can be seen, the labyrinth, spongiotrophoblast layer, and the TGC layer (**E**). By day 14.5 the mature placenta has formed (**F**). The labyrinth contains fetal capillaries and maternal blood sinuses surrounded by trophoblasts. The spongiotrophoblast layer gives rise to trophoblast glycogen cells which invade the maternal decidua interstitially, while the TGCs invade endovascularly. Modified from (Watson and Cross 2005).

1.3 Insulin-Like Growth Factor Family

1.3.1 IGFs

The Insulin-Like Growth Factor (IGF) family has been implicated in both fetal and placental development. IGFs are single-chain polypeptides of approximately 7.5 kDa consisting of an amino terminal B domain and an A domain that are separated by a short connecting C region as well as a short D region at the carboxy-terminus (Rinderknecht and Humbel 1978; Sara and Hall 1990). IGFs share 50% homology with pro-insulin, which also contains B, C, and A regions but has no D domain. The C region of pro-insulin is cleaved and the separated B and A chains remain joined to one another by disulfide bonds to generate mature insulin. Two different IGF isoforms have been identified, IGF-I and IGF-II, that share a sequence homology of 62% (Rinderknecht and Humbel 1978; Sara and Hall 1990; D'Ercole 1996) (Figure 1-4). IGF-II is also secreted as a biologically inactive pro-IGF-II peptide that undergoes cleavage by pro-protein convertase (PC4) to the mature form (Qiu, Basak et al. 2005). As well as being mitogenic, the IGFs promote differentiation and migration and inhibit apoptosis in a variety of cell types (Jones and Clemmons 1995). The IGF system is extremely complex and functions in a wide variety of physiological and pathological conditions in various tissues.

1.3.2 IGF Receptors

The IGFs are able to bind to several receptors, the type I receptor (IGF1R), the type II receptor (IGF2R), the insulin receptor (IR) and IGF1R/IR hybrids (Figure 1-4). The IR has low affinity for the IGFs and is known for its classic metabolic responses induced upon insulin binding. However, it has been shown that IGF-II binds with high affinity to an alternatively spliced variant of the IR, IR-A (Denley, Cosgrove et al.

2005). The IGF1R binds IGF-I with high affinity, IGF-II with much lower affinity and insulin with little or no affinity (Sara and Hall 1990; Germain-Lee, Janicot et al. 1992). The IGF1R shows structural and functional similarities with the IR (Morgan, Jarnagin et al. 1986). It consists of two extracellular α subunits and two transmembrane β subunits. Binding of IGFs to the extracellular domain results in intracellular signal transmission by autophosphorylation of tyrosine residues within the β subunit (Morgan, Jarnagin et al. 1986). In addition, there are IR/IGF1R hybrids composed of an $\alpha\beta$ heterodimer of the IR and an $\alpha\beta$ heterodimer of the IGF1R. These hybrid receptors bind IGF-I with high affinity and insulin with 10-fold lower affinity than that for the IR (Soos, Field et al. 1993).

The IGF2R lacks tyrosine kinase activity and is identical to the cation independent mannose-6 phosphate receptor (CI-M6PR) (Kiess, Blickenstaff et al. 1988). It is a single chain polypeptide consisting of a large extracellular domain (containing 15 repeat sequences), a single transmembrane region and a small cytoplasmic domain (Morgan, Edman et al. 1987). It binds IGF-II with very high affinity, and IGF-I and insulin with little or no affinity (Jones and Clemmons 1995; Nissley 1999). Binding to the IGF2R/M6PR was initially thought to result in clearance of IGF-II from blood and tissue as IGF2R is known to mediate the endocytosis of IGF-II and its subsequent degradation in lysosomes. This is supported by a study using mice deficient in *Igf2r* which have increased serum and tissue levels of IGF-II (Ludwig, Eggenschwiler et al. 1996). However, although the IGF2R lacks a tyrosine kinase domain, it has also been proposed that it may interact with heterodimeric G proteins to induce signal transduction (Ludwig, Le Borgne et al. 1995). Indeed, it has been shown that the IGF2R can interact with and activate G_i proteins when stimulated by IGF-II, but not mannose-6 phosphate (Murayama, Okamoto et al. 1990; Okamoto and Nishimoto

1991) and this is required for IGF-II - IGF2R dependent signaling (Ikezu, Okamoto et al. 1995).

1.3.3 IGF Binding Proteins

The bioavailability and biological actions of the IGFs are regulated by a family of 6 IGF binding proteins (IGFBPs 1-6) (Figure 1-4). IGFBPs have conserved N- and C-terminal regions and bind to the IGFs with high affinity (Baxter 2000; Mohan and Baylink 2002; Duan and Xu 2005). IGFBP-6 differs from the other IGFBPs in that it lacks two conserved cysteine residues in the N-terminal domain and it has a markedly higher affinity for IGF-II than for IGF-I, whereas the other IGFBPs bind the two IGFs with relatively similar affinities (Baxter 2000). In blood, about 75% of IGFs circulate as a complex with IGFBP-3 or IGFBP-5 and an acid-labile subunit (ALS). This large complex acts as a reservoir, it is unable to cross the endothelium and has a half life of 15-20 h (Mohan and Baylink 2002). The remaining IGFs in the circulation bind to one of the remaining IGFBPs enabling them to cross the endothelium increasing their availability to local tissues.

Binding of IGFs to IGFBPs can result in either inhibition or enhancement of IGF action. The affinity of IGFBPs for IGFs is higher than that of the receptors for IGFs, therefore binding of IGFBPs to IGFs prevents interactions with the IGF receptors (Clemmons 1998; Baxter 2000; Mohan and Baylink 2002; Rosenzweig 2004; Duan and Xu 2005). Conversely, several studies have also shown that a number of IGFBPs are able to stimulate IGF actions in a variety of cell types. Indeed, the same IGFBP could act to enhance or inhibit IGF actions depending on the cell type, amount of IGFBP and post-translational modifications such as glycosylation or phosphorylation, which result in altered affinity for IGFs (Baxter 2000; Mohan and

Baylink 2002; Duan and Xu 2005). Several IGFBPs have also been known to be associated with the cell surface or ECM which reduces their affinity for the IGFs and hence can also modulate IGF actions (Clemmons 1998; Baxter 2000; Mohan and Baylink 2002). Several different types of proteases cleave IGFBPs, resulting in complete or partial reduction of their ability to bind the IGFs (Clemmons 1998; Baxter 2000; Mohan and Baylink 2002). IGFBP related proteins (IGFBPrP) share structural homology to the N-terminal region of the IGFBPs and bind IGFs with low affinity (Mohan and Baylink 2002). Their functional significance is not well understood. Interestingly, IGFBPs have also been shown to have IGF-independent functions (Mohan and Baylink 2002; Duan and Xu 2005).

NOTE: This figure is included on page13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-4 The insulin-like growth factor (IGF) system The IGF system consists of IGF-I, IGF-II, IGF binding proteins (IGFBPs) 1-6, the IGF receptors (IGF1R and IGF2R) and the insulin receptor (IR). The IGFBPs must be cleaved from the IGFs in order to facilitate binding of IGFs to their receptors. ALS = acid labile subunit. Diagram from (Gicquel and Le Bouc 2006).

1.4 The IGF System in Pregnancy

Maternal circulating concentrations of IGF-I and IGF-II increase during early pregnancy in humans (Wilson, Bennett et al. 1982; Gargosky, Moyse et al. 1990; Hills, English et al. 1996), suggesting that IGFs play an endocrine role in the regulation of placental and fetal growth and maternal adaptation to pregnancy. However, IGF-II is the most abundant IGF in the maternal circulation during pregnancy in humans (Gargosky, Moyse et al. 1990).

1.4.1 IGFs in Preimplantation Development

Insulin and IGF-I are not detectable in mouse preimplantation embryos whereas IGF-II is first expressed at the 2-cell stage of mouse development, when transcription from the embryonic genome is activated (Rappolee, Sturm et al. 1992; Schultz, Hogan et al. 1992; Schultz, Hahnel et al. 1993). However, the expression of IGF-II is significantly reduced and delayed until at least the 8-cell stage of development following *in vitro* fertilization (IVF) and culture of mouse embryos (Stojanov, Alechna et al. 1999). The *Igf1r* and the *Insr* are not detected in the mouse embryo until the 8-cell stage, whereas *Igf2r* mRNA, like *Igf2*, is first expressed at the 2-cell stage of mouse development (Harvey and Kaye 1991; Rappolee, Sturm et al. 1992; Schultz, Hogan et al. 1992; Schultz, Hahnel et al. 1993; Markham and Kaye 2003).

Mouse embryos cultured in media enriched with insulin, IGF-I or IGF-II produced significantly more blastocysts and significantly more hatching blastocysts than those in control media (Harvey and Kaye 1992; Harvey and Kaye 1992; O'Neill 1997; Kurzawa, Glabowski et al. 2001; Lin, Yen et al. 2003; Pantaleon, Jericho et al. 2003). In addition, both IGF-I and IGF-II have been shown to increase glucose uptake by the blastocyst and increase total blastocyst and ICM cell numbers (Harvey and Kaye

1992; Harvey and Kaye 1992; Rappolee, Sturm et al. 1992; Pantaleon and Kaye 1996; O'Neill 1997; Kurzawa, Glabowski et al. 2001; Pantaleon, Jericho et al. 2003; Glabowski, Kurzawa et al. 2005). Interestingly, IGF-II, but not IGF-I, also increases trophectoderm (TE) cell numbers (Harvey and Kaye 1992; O'Neill 1997; Pantaleon, Jericho et al. 2003). Conversely, mouse embryos cultured with antisense IGF-II oligonucleotides had delayed blastocyst formation, and 20% fewer cells (Rappolee, Sturm et al. 1992). However, Leu^{27} IGF-II, which only binds to the IGF2R, did not increase blastocyst cell numbers, suggesting that IGF-II effects on cell proliferation were mediated by the InsR or the IGF1R (Rappolee, Sturm et al. 1992). However, IGF-II causes significant increases in cleavage rates and stimulates glucose transport in 2-cell embryos, only when the IGF2R is present, suggesting that the IGF2R is functional during early embryo development (Pantaleon, Jericho et al. 2003).

The IGFs have also been shown to play a role in preventing apoptosis (Byrne, Southgate et al. 2002; Fabian, Il'kova et al. 2004; Glabowski, Kurzawa et al. 2005) and from oxidative stress in mouse embryos *in vitro* (Kurzawa, Glabowski et al. 2001). Treatment of the embryo with low concentrations of IGF-I result in significantly higher implantation rates following transfer. However, high preimplantation IGF-I concentrations *in vitro* or *in vivo* have been shown to decrease IGF1R protein, decrease insulin-stimulated glucose uptake, increase apoptosis in the ICM, increase resorption rates and decrease fetal weight in mice (Chi, Schlein et al. 2000; Pinto, Schlein et al. 2002; Eng, Sheridan et al. 2007). This may represent a possible mechanism for increased rates of miscarriage in women with polycystic ovary syndrome (PCOS) (Sagle, Bishop et al. 1988; Balen, Tan et al. 1993; Tulppala, Stenman et al. 1993) in whom elevated insulin and IGF-I occur in blood. To date

there have been no studies that examined the effects of preimplantation IGF-II treatment on subsequent pregnancy outcomes.

1.4.2 IGF Expression in the Placenta

IGFs

Igf1 mRNA is not detectable in any of the tissues of fetal origin in the human placenta during the first trimester, but is expressed in low levels in the mesodermal cells of the chorionic villi (Han, Bassett et al. 1996; Lacey, Haigh et al. 2002). *Igf2* is differentially expressed in the two layers of the villous trophoblast in the placenta. The inner layer of CTB express *Igf2*, while the outer STB is negative, suggesting that *Igf2* is down-regulated in association with trophoblast differentiation (Ohlsson, Holmgren et al. 1989; Redline, Chernicky et al. 1993; Han, Bassett et al. 1996). Abundant *Igf2* mRNA has been identified in the columns of CTBs and EVTs invading into the maternal endometrium, including those invading the maternal blood vessels (Ohlsson, Larsson et al. 1989; Hamilton, Lysiak et al. 1998; Gratton, Asano et al. 2002), and actually increases as the cells invade into the decidua (Han, Bassett et al. 1996).

Placentas from women with preeclampsia were found to have increased *Igf2* mRNA expression in trophoblasts surrounding placental infarcts suggesting a local role for IGF-II in placental repair or remodelling (Gratton, Asano et al. 2002). However, total *Igf2* mRNA and protein from term placenta was significantly reduced in preeclamptic women, suggesting that IGF-II might be associated with impaired trophoblast invasion leading to the pathogenesis of preeclampsia (Shin, Lee et al. 2003). In addition, abnormal processing of IGF-II to its mature form may also play a role in impaired placentation. Inhibitors of proprotein convertase -4 (PC4) block IGF-II processing and decrease trophoblast migration (Qiu, Basak et al. 2005). Interestingly,

women with IUGR pregnancies have elevated circulating biologically inactive pro-IGF-II concentrations (Qiu, Basak et al. 2005).

IGF-II is expressed in the entire mouse placenta, in both the junctional zone and labyrinth from days 9.5 - 18.5 (Redline, Chernicky et al. 1993; Han and Carter 2000; Tycko and Efstratiadis 2002; Carter, Nygard et al. 2006). After this, IGF-II expression in the labyrinthine trophoblast remains constant but expression of IGF-II in the spongiotrophoblast decreases significantly as the new trophoblast glycogen cell population emerges within it (days 12.5 - 15.5) (Redline, Chernicky et al. 1993; Carter, Nygard et al. 2006). Glycogen cells, which are homologous to human intermediate trophoblast, strongly express IGF-II (Redline, Chernicky et al. 1993). The trophoblast lining the area of maternal-fetal exchange, the labyrinth, on the other hand, maintained a constitutive lower level of IGF-II expression throughout late pregnancy (Redline, Chernicky et al. 1993).

IGF Receptors

Human trophoblast cells express both IGF receptors, the IR and IGF1R-IR hybrids (Soos, Field et al. 1993). In the first trimester, both *Igf1r* and *Igf2r* mRNA have been localized to villous CTBs with the highest expression in secondary villi (Ohlsson, Holmgren et al. 1989). However, it was noted that they may have a much broader expression pattern that was unable to be detected in this study. Similarly, IGF1R protein was localized to all cell types of the term placenta except in villous endothelium (Hayati, Cheah et al. 2007). In addition, one study of full term placenta has been undertaken to determine whether the microvillous membranes, which are bathed by the maternal intervillous circulation, and basal plasma membrane, which is adjacent to the endothelium of fetal capillaries, have binding sites for IGF-II

(Rebourcet, de Ceuninck et al. 1998). Both membranes contained three major (250, 135, and 130 kDa) ^{125}I-IGF-II binding site complexes. The 250 kDa band (IGF2R) was the main band in the basal plasma membranes, the 135-kDa band (IR α subunit) was the main one in the microvillous membranes and both membranes contained the 130-kDa band (IGF1R α subunit) (Rebourcet, de Ceuninck et al. 1998).

In mice, IGF2R is strongly expressed on days 14.5 – 16.5 of gestation in the junctional zone in glycogen cells, spongiotrophoblasts and secondary TGCs, and in the fetal endothelium in the labyrinth (Coan, Conroy et al. 2006). To date there have been no studies localizing IGF1R to the mouse placenta nor localizing IGF2R in early pregnancy.

IGFBPs

In the placenta, IGFBPs play a role in supporting implantation, regulating the extent of decidualisation and modulating local levels of the IGFs (Slater and Murphy 1999). In humans, *Igfbp-1* mRNA is abundant in the decidual stromal cells by 10-12 weeks (Han, Bassett et al. 1996; Gratton, Asano et al. 2002) and is therefore spatially and temporally positioned to play a key role in regulating IGF activity at the feto-maternal interface (Crossey, Pillai et al. 2002). Human decidualised endometrium produces both phosphorylated and non-phosphorylated forms of IGFBP-1 (Gibson, Aplin et al. 2001). However, trophoblasts can de-phosphorylate IGFBP-1 and non-phosphorylated IGFBP-1 is preferentially produced when IGF-II is present (Gibson, Aplin et al. 2001). Non-phosphorylated IGFBP-1 has a decreased affinity for IGF-I and would therefore increase IGF bioavailability. Interestingly, placental IGFBP-1 levels are significantly increased in women with preeclampsia (Shin, Lee et al. 2003), whilst the expression of IGFBP-1 in the decidua is significantly decreased (Gratton,

Asano et al. 2002). *Igfbp-5* mRNA is also detected in some of the decidualised stromal cells but is relatively less abundant than *Igfbp-1* mRNA (Han, Bassett et al. 1996). Other IGFBP mRNAs (*Igfbp-2*, *bp-3*, *bp-4*, and *bp-6*) are detectable in the decidual stromal cells in the latter part of the first trimester (Han, Bassett et al. 1996) and are expressed in variable abundance in decidual cells during the second and third trimesters. However, *Igfbp-1* mRNA is the most abundant during this time and is expressed in most decidual cells but is absent in any of the cells of fetal origin (Han, Bassett et al. 1996). *Igfbp-3* mRNA is the only IGFBP expressed in the chorionic villi during the first trimester and it continues to be expressed in the mesodermal core of placental villi throughout pregnancy.

In mice, *Igfbp-2* mRNA is expressed weakly in the spongiotrophoblast and glycogen cells after day 10.5 of gestation (Han and Carter 2000; Carter, Nygard et al. 2006). *Igfbp* mRNAs, except *Igfbp-4*, are not expressed in the mouse decidua. However, *Igfbp-3*, *-4* and *-5* are expressed in endothelium of maternal vessels, and *Igfbp-2* and *-6* in the myometrium (Han and Carter 2000; Carter, Nygard et al. 2006). Thus in rodents it is less apparent that IGF-II and IGFBPs interact at the interface between trophoblast and decidua. However, such an interaction may be important in regulating trophoblast invasion of the maternal blood vessels.

1.4.3 Roles of IGFs in Placentation

Trophoblast derived IGF-II has been regarded to play an important role in placental development. In particular, IGF-II has been shown to promote migration of human EVT cells (Irving and Lala 1995; Hamilton, Lysiak et al. 1998; McKinnon, Chakraborty et al. 2001) and increase cell numbers in first trimester villous cytotrophoblasts *in vitro* (Hills, Elder et al. 2004). IGF-II effects on promoting trophoblast migration of

EVT cells *in vitro* were found to be mediated by the IGF2R using a Gi-coupled receptor and mitogen activated protein kinase (MAPK) intracellular signalling pathway (McKinnon, Chakraborty et al. 2001).

1.4.4 IGF gene mutation studies

IGF-II is a paternally expressed imprinted gene and therefore there has been a great deal of discussion surrounding its role in the genetic conflict theory. This theory predicts that paternally expressed imprinted genes, such as IGF-II, promote fetal growth, and maternally expressed genes inhibit fetal or placental growth (Tycko and Efstratiadis 2002). Research thus far has been consistent with this hypothesis.

It was previously shown in mice that on day 15 *Igf2* null placentas were 51% of the weight of the wild type placentas (Lopez, Dikkes et al. 1996). On average the null placentas had approximately 44% fewer cells than those found in the wild-type placenta. Upon further investigation it was discovered that the *Igf2* null placenta had fewer glycogen cells and less placental glycogen than wild-type controls (Lopez, Dikkes et al. 1996). However, since glycogen cells are a highly invasive trophoblast cell population that are suggested to be involved in remodelling maternal arterioles in the second half of gestation (Adamson, Lu et al. 2002), it may be that deletion of *Igf2* reduces vascular remodeling and thereby maternal blood flow to the placenta. Heterozygous mice carrying an *Igf2* gene disrupted by targeting were born approximately 60% of the size of their wild-type littermates (DeChiara, Efstratiadis et al. 1990) (Figure 1-5). It was therefore proposed that intrauterine growth retardation of the *Igf2* null mouse is due to either decreased glucose transport or impaired glycogen metabolism.

In mice, deletion of an *Igf2* transcript (P0) specifically expressed in the trophoblasts of the labyrinthine layer from the P0 promoter of the *Igf2* gene leads to reduced placental and fetal growth (Constancia, Hemberger et al. 2002; Sibley, Coan et al. 2004). Passive permeability of nutrients in the mutant placenta is decreased but secondary active placental amino acid transport is initially upregulated, compensating for the decrease in passive permeability. Later the compensation fails and fetal growth restriction ensues (Constancia, Hemberger et al. 2002). At birth the *Igf2* P0 mutant pups were approximately 69% of normal birth weight (Constancia, Hemberger et al. 2002; Sibley, Coan et al. 2004). This was followed by postnatal 'catch-up' growth, which was complete by three months of age (Constancia, Hemberger et al. 2002). Interestingly, the degree and time of onset of placental growth deficiency in P0 mutants was identical to that in *Igf2* null mice, which lack the IGF-II peptide in all placental layers, as well as the fetus. Also, analyses of the transfer of three inert hydrophilic solutes (^{14}C-mannitol, ^{51}CrEDTA, and ^{14}C-inulin) revealed a reduction in permeability in P0 mutant placentas, the surface area for exchange in the labyrinth was reduced, and the thickness of the barrier for exchange was increased in P0 fetuses compared to wild type (Sibley, Coan et al. 2004). As a result, the average theoretical diffusing capacity in P0 knockout placentas was dramatically reduced to 40% of that of wild-type placentas (Sibley, Coan et al. 2004). These findings show that the labyrinthine trophoblast specific P0 transcript is critical for sustaining normal growth of the placenta and is essential in attaining normal surface area and thickness of trophoblasts in the labyrinthine layer where solute exchange takes place.

Previous studies have undertaken developmental analyses of growth kinetics in mouse embryos carrying null mutations of the genes encoding *Igf1*, *Igf2* and *Igf1r*, alone or in combination, and were able to define the onset of mutational effects

leading to growth deficiency (Baker, Liu et al. 1993; Liu, Baker et al. 1993). Since *Igf2* is a paternally expressed imprinted gene mice deficient in the paternal *Igf2* gene were used (p-). Starting at day 13.5 the three classes of mutant embryos (the *Igf2*(p-) mutant, *Igf1r*(-/-), and the *Igf2*(p-)/*Igf1r*(-/-) double mutant) became distinct in size and displayed a decrease in growth rate in comparison with wild-type controls resulting in the respective relative weights of these embryos being approximately 30%, 45%, and 60% of normal at day18.5 (Baker, Liu et al. 1993; Liu, Baker et al. 1993) (Figure 1-5). *Igf1* null mice exhibited a growth deficiency similar to that observed in *Igf2* mutants (60% of normal birth weight) (Liu, Baker et al. 1993). Comparison of the placental growth curves revealed that placental growth was impaired in the *Igf2*(p-) mutation but neither the *Igf1*(-/-) nor the *Igf1r*(-/-) mutation affected placental weight (Baker, Liu et al. 1993). *Igf1*(-/-)/*Igf1r*(-/-) double mutants did not differ in phenotype from *Igf1r* single mutants, suggesting that the IGF-I ligand does not utilise any other receptor other than IGF1R (Liu, Baker et al. 1993).

Murine embryos that inherit a non-functional *Igf2r* gene from their fathers are viable and develop normally to adults. However, the majority of mice inheriting the same mutated allele from their mothers die around birth as a consequence of major cardiac abnormalities. These mice have increased serum levels and tissue levels of IGF-II and IGFBPs, exhibit overgrowth (130% of normal birth weight) and generalised organomegaly and have increased placental weight, kinky tail and oedema (Lau, Stewart et al. 1994; Ludwig, Eggenschwiler et al. 1996). These demonstrate that regulation of IGF-II levels by IGF2R is essential to prevent fetal overgrowth. While lack of either IGF ligand or elimination of the IGF1R are dwarfing mutations for the embryo, only the absence of IGF-II causes a decrease in placental size (Baker, Liu et al. 1993). This can be attributed to different ligand/receptor signalling systems

operating in the two components of the conceptus. On the basis of this genetic evidence, it has been postulated that IGF-II is involved in placental growth via an interaction with an unknown placental receptor (XR), which is possibly the IR (Baker, Liu et al. 1993; Liu, Baker et al. 1993; Ludwig, Eggenschwiler et al. 1996), and that appears to be distinct from the XR acting on the embryo proper. The likely presence of an XR was deduced from the observation that the growth deficiency of *Igf1r/Igf2* double mutants is more severe than the phenotype manifested by either mutation acting alone (Liu, Baker et al.1993).

NOTE: This figure is included on page23 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-5 Disruption of one or a combination of IGF genes affects fetal growth in mice. Fetal growth is expressed as a percentage of normal body weight. m- = maternally disrupted allele; p- = paternally disrupted allele; -/- = both alleles disrupted. Diagram from (Gicquel and Le Bouc 2006).

1.5 Matrix Degrading Enzymes

Invasive human trophoblast cells also secrete an array of proteolytic enzymes among which are certain metalloproteinases (MMPs). These enzymes are the only ones capable of digesting the different constituents of the endometrial basement

membrane and ECM (several collagen types, laminin, fibronectin) and are therefore considered rate limiting in trophoblast invasion (Bischof, Meisser et al. 1998). MMPs are normally secreted as inactive pre-forms that must first be activated by proteases. Normally MMP activity is counterbalanced by proteins termed tissue inhibitors of metalloproteinases (TIMPs) (Lala and Graham 1990; Bischof, Martelli et al. 1995; Bass, Li et al. 1997).

Expression of MMP-1 and MMP-2 in the invasive phenotype of EVT has been found throughout pregnancy (Huppertz, Kertschanska et al. 1998; Bjorn, Hastrup et al. 2000). MMP-3 is expressed in the proliferative phenotype and in the transitional stages toward the invasive phenotype with a slight tendency to increase as invasion proceeds. MMP-9 is expressed by EVTs in early pregnancy but is down-regulated towards the end of gestation (Huppertz, Kertschanska et al. 1998). Similarly, as trophoblasts differentiate along the invasive pathway they up-regulate both MMP-9 and TIMP-3 mRNA and protein to regulate the depth of trophoblast invasion (Bass, Li et al. 1997). In contrast, Isaka et al. observed MMP-2 expression in EVTs, and MMP-9 mainly in villous CTB (Isaka, Usuda et al. 2003). In mice, the fact that MMP-2 and -9 are expressed in deicudal stromal cells and MMP-9 is expressed in TGCs, coupled with the expression of TIMP-3 in the stroma surrounding the embryo, suggests that the balance between MMPs and TIMPs may also regulate trophoblast invasion in the murine uterus (Das, Yano et al. 1997; Bany, Harvey et al. 2000; Whiteside, Jackson et al. 2001).

There have been several studies that examined the role of MMPs in CTB invasion and both MMP-9 and MMP-2 have been implicated (Fisher, Cui et al. 1989; Librach, Werb et al. 1991; Bischof, Martelli et al. 1995; Isaka, Usuda et al. 2003).

Metalloproteinase inhibitors, and a function-perturbing antibody specific for MMP-9, completely inhibit CTB invasion, whereas inhibitors of the plasminogen activator system had only a partial (20-40%) inhibitory effect (Librach, Werb et al. 1991). Also, invasion of early CTBs was inhibited by TIMP-2 and MMP-2 antibodies (Isaka, Usuda et al. 2003). Studies in rodents have yielded similar results. Rcho-1 trophoblast cell cultures and rat EPC outgrowths display increased expression of MMP-9 accompanying cell differentiation into an invasive phenotype (Peters, Albieri et al. 1999). Also, murine embryos exposed to MMP-9 anti-sense oligonucleotides or exogenous TIMP-3, exhibited reduced ECM-degrading activity which was correlated with diminished MMP-9 activity (Whiteside, Jackson et al. 2001).

In addition, MMPs and TIMPs have been shown to interact with the IGF system. IGF-I, IGF-II and IGFBP-1 can increase levels of MMP-2 and MMP-9 in villous cytotrophoblast cells (Hills, Elder et al. 2004) and IGF-I and IGF-II can reduce TIMP-3 mRNA expression in decidualised stromal cells (Irwin, Suen et al. 2001). Also, overexpression of TIMP-1 has been shown to increase levels of IGFBP-1 protein and to subsequently decrease IGF-II levels and IGF1R signalling in a transgenic mouse hepatic tumour model (Martin, Fowlkes et al. 1999).

1.6 uPA and Plasminogen

Urokinase plasminogen activator (uPA), the uPA receptor (uPAR) and uPA inhibitors play a central role in trophoblast migration and invasion. EVT cells have been shown to exhibit a highly polarised distribution of uPAR bound uPA at the invasive front (Multhaupt, Mazar et al. 1994) (Figure 1-6). uPA is secreted as an inactive pro-enzyme (pro-uPA) which, upon binding to its specific receptor (uPAR) on the cell surface, is cleaved to its active form. Following activation, cell bound UPA can

catalyse the conversion of the inactive zymogen plasminogen, to the active proteinase plasmin (Kjoller, Kanse et al. 1997) which can then go on to activate MMPs and directly degrade certain ECM components required for invasion. However, invasion is tightly regulated by plasminogen activator inhibitor-1 (PAI-1) which controls proteolysis and remodelling of maternal tissue during trophoblast invasion (Yebra, Parry et al. 1996; Floridon, Nielsen et al. 2000). Alternatively, the uPA:uPAR interaction can stimulate EVT migration independently of uPA catalytic action, using the mitogen activated protein kinase (MAPK) pathway and calcium signalling events requiring phosphatidylinositol-3 kinase (PI3K) and phospholipase C (PLC) (Liu, Chakraborty et al. 2003).

NOTE: This figure is included on page26 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-6 Diagram representing the polarized expression of uPAR and PAI-1 in migrating a cell

uPA is expressed by invading and migrating trophoblast cells throughout early human pregnancy in agreement with their role in embryo implantation. Transcripts for uPA are first detectable at the blastocyst stage in mice and are expressed in the EPC on days 6.5 - 7.5, TGCs from days 10.5 – 13.5 and glycogen cells from days 12.5 - 15.5

(Sappino, Huarte et al. 1989; Harvey, Leco et al. 1995; Teesalu, Blasi et al. 1996; Teesalu, Blasi et al. 1998; Teesalu, Masson et al. 1999). Interestingly, endometrial uPA activity also increases toward the time of implantation (Aflalo, Sod-Moriah et al. 2004). Conversely, transcripts for uPAR shows strong expression in the oocyte and 1-cell embryo, decreased abundance at the 2-cell stage and re-accumulation in morulae and blastocysts (Harvey, Leco et al. 1995). *upar* mRNA is weakly expressed in trophoblasts and decidual tissue on days 10.5 and 11.5 of gestation (Teesalu, Blasi et al. 1998).

Similarly, *upa* mRNA is expressed in human blastocysts (Khamsi, Armstrong et al. 1996) and, along with its receptor the uPAR, has been localised to human placenta (Multhaupt, Mazar et al. 1994; Floridon, Nielsen et al. 1999). During the first trimester, uPAR protein was observed on non-proliferating trophoblasts within cell columns whereas uPA was observed in anchoring distal trophoblasts and in the EVT invading the interstitium. uPA and uPAR protein were both present in the endovascular trophoblasts. As CTB mature and differentiate into STB their expression of uPAR mRNA diminishes, coinciding with the decreased invasiveness of trophoblasts later in gestation (Zini, Murray et al. 1992). PAI-1 protein is expressed predominantly by the maternal decidua but also by the villous STB as well as EVT and this is thought to control trophoblast invasion (Feinberg, Kao et al. 1989; Zini, Murray et al. 1992; Floridon, Nielsen et al. 2000). Indeed, elevated levels of PAI-1 protein have been correlated with decreased migration of trophoblasts from first trimester placental explants (Bauer, Pollheimer et al. 2004).

uPA, uPAR and plasminogen are important in attachment and implantation of the blastocyst. Inhibition of uPA decreases the extent of trophoblast outgrowth and

attachment *in vitro* (Kubo, Spindle et al. 1981). Similarly, embryos homozygous for the mutation t^{w73} have reduced levels of plasminogen activators and do not implant *in vivo* (Axelrod 1985). Interestingly, *in vitro* development reduces uPA protein expression and activity in rat embryos (Aflalo, Sod-Moriah et al. 2004; Aflalo, Sod-Moriah et al. 2005). Plasmin and plasminogen have also been shown to improve development to the blastocyst stage and improve attachment and subsequent outgrowth in mouse embryos (Menino and O'Claray 1986), whilst improving hatching rates in ovine embryos (Menino, Dyk et al. 1989). This may be due to the ability of plasmin to proteolytically degrade the zona pellucida, as seen in bovine embryos (Cannon and Menino 1998).

upa null mice have decidual extravascular fibrin deposits which are not present in wild type placentas but the extent of trophoblast invasion is unchanged (Teesalu, Blasi et al. 1998). Similarly, *upa/mmp-11* double knockouts display no differences in trophoblast invasion or overall placental morphology and are viable (Teesalu, Masson et al. 1999). Also, *upa*r -/- mice are born and survive to adulthood with no obvious phenotypic abnormalities but the placentas of these mice have not been assessed (Bugge, Suh et al. 1995). Conversely, plasminogen (*Plg*) deficient mice that are treated with an MMP inhibitor, display improper vascularisation and development of the placental labyrinth, abnormal distribution of TGCs and increased perinatal mortality (Solberg, Rinkenberger et al. 2003). This suggests that plasmin and MMP activities are both involved in placental development and that there is some functional overlap between the two, since these effects were not seen in control mice treated with MMP inhibitors or in *Plg*-deficient mice alone (Bugge, Flick et al. 1995; Solberg, Rinkenberger et al. 2003). Therefore, the uPA system appears to play important, but not essential, roles in blastocyst implantation and placental

development since many of the null mice have no defects in implantation or placentation.

1.7 IGF and uPA System Interactions

Over the past decade a novel interaction between the IGF and UPA systems has been identified that may play an important role in regulating trophoblast migration. It has been shown that IGF2R can bind uPAR at sites different from that used for IGF-II binding or uPA binding and is part of a large plasma membrane complex (Nykjaer, Christensen et al. 1998; Godar, Horejsi et al. 1999; Kreiling, Byrd et al. 2003; Olson, Yammani et al. 2004). In this way, IGF2R is able to modulate the subcellular distribution of uPAR and is capable of directing it to lysosomes (Nykjaer, Christensen et al. 1998). In addition, IGF2R binds to plasminogen and controls the conversion of plasminogen to plasmin (Godar, Horejsi et al. 1999; Olson, Yammani et al. 2004). IGF-II has also been shown to increase the activity of surface uPAR, and IGF-II induced cell migration is dependent on uPAR, uPA and plasmin activity (Gallicchio, Kaun et al. 2003). Interestingly, plasmin is able to dissociate IGF-I and IGF-II from IGFBPs (Campbell, Novak et al. 1992), suggestive of a positive feedback loop. It was therefore hypothesised that binding of IGF-II to the IGF2R may increase IGF2R/uPAR interactions and/or increase uPA binding to uPAR, resulting in increased plasminogen conversion to plasmin at the cell surface to increase cell migration in a rhabdomyosarcoma cell line (Gallicchio, Kaun et al. 2003). We hypothesise that this is the mechanism by which IGF-II promotes blastocyst implantation.

NOTE: This figure is included on page30 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1-7 Diagram representing the IGF2R and uPAR interaction on the cell membrane. IGF2R is able to interact with uPAR to form part of a large plasma membrane complex involving IGF-II, uPA and plasminogen. Upon the formation of this complex plasminogen is converted to its active form plasmin. Plasmin can then go on to activate MMPs and increase cell invasion. Modified diagram by Roberts, CT.

1.8 Oxygen

In 1987, Hustin and Schaaps reported that blood flow to the IVS was not fully established until approximately 11 to 12 weeks of gestation (Hustin and Schaaps 1987). Therefore a very limited amount of maternal blood reaches the IVS during the first trimester. Further research and the use of Doppler ultrasound during pregnancy confirmed that there is very little blood flow within the IVS until 11 to 12 weeks of gestation (Foidart, Hustin et al. 1992; Coppens, Loquet et al. 1996). It was found that this lack of blood flow in the first trimester is due to CTBs initially plugging the maternal spiral arterioles. However, at about 11 weeks of gestation the plugs are displaced and blood flow commences (Hustin and Schaaps 1987; Burton, Jauniaux et al. 1999). As a result, the oxygen tension within the IVS *in vivo* rises steeply from 18 mmHg (2%) at 8 weeks to approximately 60 mmHg (8%) at 12 weeks (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). Therefore it can be concluded that the human embryo and placenta develop in an oxygen poor environment in the first trimester compared with the later stages (Rodesch, Simon et al. 1992).

In mice, maternal blood flow to the placenta is not established until day 10 of pregnancy (Muntener and Hsu 1977), upon the formation of the hemochorial chorioallantoic placenta. To date, uterine oxygen tension during pregnancy in mice has not been measured, and there is no evidence to suggest that trophoblasts ‘plug’ the spiral arterioles as is the case in humans. Therefore, the oxygen tension in the murine conceptus is unknown.

Some studies suggest that culture of HTR-8/SVneo cells (a human first trimester cytotrophoblast cell line) in low (1%) oxygen conditions increases uPAR expression

on the cell surface and promotes invasion of these cells when compared to culture under standard conditions (20% oxygen) (Graham, Fitzpatrick et al. 1998) and upregulates the expression of PAI-1 and IGFBP-3 (Fitzpatrick and Graham 1998; Koklanaris, Nwachukwu et al. 2006). In contrast, there has also been a report that culture of HTR-8/SVneo cells in 2% oxygen decreases trophoblast invasion and increases cell proliferation compared to those cultured in 20% oxygen (Kilburn, Wang et al. 2000). The presence of serum, the addition of plasminogen, the reduced oxygen levels, or the use of a different invasion assay in the first study may account for the different results observed. There have also been studies utilising first trimester villous explants, where culture in 2% oxygen inhibited CTB cell invasion in 10-12 week villous explants (Genbacev, Joslin et al. 1996) and stimulated proliferation in 6-8 week anchoring villi (Genbacev, Zhou et al. 1997). Similarly, it has been shown by a separate group that culture in 3% oxygen of 5 to 8 week villous explants increases EVT outgrowth associated with increased cell proliferation, fibronectin synthesis and MMP-2 activity (Caniggia, Mostachfi et al. 2000). In disagreement with these reports is a recent study where culture of 8-12 week villous explants in 1.5% oxygen resulted in fewer explants producing EVT outgrowths and a decrease in the area of outgrowth and number of cells that these explants produced (James, Stone et al. 2006). In addition, utilising a different *in vitro* technique involving culture of myometrial spiral arteries with fluorescently-labelled EVTs, culture in 3% oxygen suppressed EVT invasion regardless of the route (interstitial or endovascular) compared to those cultured in 17% oxygen (Crocker, Wareing et al. 2005). However, there was more endovascular EVT invasion than interstitial invasion in 17% oxygen.

Unfortunately, there has been very little research that has examined the effect of low oxygen on trophoblast behaviour in rodents. This may be an important area for future

research, especially considering the broad use of murine knockouts to investigate hypoxia regulated genes. However, it is known that culture of Rcho-1 cells derived from rat transplantable choriocarcinomas in 1% oxygen, inhibits differentiation to TGCs and maintains a stem cell-like state (Gultice, Selesniemi et al. 2006).

1.9 Hypoxia Inducible Factors

One of the most important factors in the cellular response to hypoxia is hypoxia inducible factor (HIF). HIF belongs to the basic-helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors (Kozak, Abbott et al. 1997; Wenger and Gassmann 1999; Semenza 2000) and is involved in the upregulation of genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT-1) and various glycolytic enzymes, which play critical roles in erythropoiesis, angiogenesis, glucose transport and glycolysis (Wenger and Gassmann 1999; Safran and Kaelin 2003). Interestingly, PAI-1 (Zhang, Wu et al. 2004; Carroll and Ashcroft 2006; Meade, Ma et al. 2007), IGF-II (Feldser, Agani et al. 1999) and uPAR (Carroll and Ashcroft 2006) are also HIF target genes. HIF-1 is a heterodimer consisting of a HIF-1α subunit bound to the aryl hydrocarbon nuclear translocator (ARNT), which is also known as HIF-1β (Wang and Semenza 1995; Safran and Kaelin 2003). HIF-1α is unique to HIF-1, whereas HIF-1β is a common subunit for several bHLH/PAS transcription factors (Iyer, Leung et al. 1998). Results have suggested that ARNT has a higher affinity for the nuclear compartment under hypoxia (in the presence of HIF-1α) than in normoxia (in the absence of HIF-1α) (Chilov, Camenisch et al. 1999). Thus, HIF-1α:ARNT heterodimer formation occurs mainly in the nucleus (Chilov, Camenisch et al. 1999). Also, upon dimerisation with HIF-1α, ARNT produced an allosteric change in the conformation of its partner factor to generate a DNA binding complex (Kallio, Pongratz et al. 1997). Results suggest

that the PAS domain of ARNT is critical in forming complexes with HIF-1α (Kallio, Pongratz et al. 1997). Upon hypoxia induced nuclear translocation and dimerisation, HIF is able to bind to the hypoxia response element (HRE) of target genes, which has the core consensus sequence 5'-CGTG-3', in responsive genes (Rajakumar and Conrad 2000). In mammals, three genes have been shown to encode HIF-α subunits. The expression of HIF-1α is ubiquitous whereas the expression of its paralogs, HIF-2α (also known as endothelial PAS domain protein 1, HIF-1-like factor, HIF-related factor, and member of PAS superfamily 2) (Ema, Taya et al. 1997; Flamme, Frohlich et al. 1997; Tian, McKnight et al. 1997) and HIF-3α (Gu, Moran et al. 1998) is much more restricted (Wenger and Gassmann 1999; Semenza 2000; Daikoku, Matsumoto et al. 2003). This suggests that HIF-1 plays a general role in homeostasis whereas HIF-2 and HIF-3 may play more specialised roles (Wenger and Gassmann 1999; Semenza 2000).

There are two different mouse HIF-1α isoforms that have two different first exons (termed I.1 and I.2) that are regulated by distinct promoters (Wenger, Rolfs et al. 1997). The predicted protein from the exon I.1 containing isoform is 12 amino acids shorter than the exon I.2 containing isoform. It has also been suggested that these two isoforms may serve different functions as HIF-1α exon I.1 is differentially expressed, being highest in mouse kidney, testis, tongue and stomach and undetectable in liver, whereas the exon I.2 isoform is ubiquitously expressed (Wenger, Rolfs et al. 1998). No such corresponding HIF-1α isoforms have been discovered in humans but a recent study has described the existence of a splice variant of HIF-1α which lacks exon 14 (sHIF-1α) in humans (Gothie, Richard et al. 2000). This isoform can compete with the full length HIF-1α for HIF-1β binding,

thereby regulating HIF mediated transcription. However, the regulation of HIF-1α alternative splicing remains unknown.

1.9.1 HIF Stabilisation

The process of HIF-1α activation has been studied extensively over the past few years and includes both enhanced protein stability and transcriptional activity. In well-oxygenated cells, HIF-1α has a half-life of less than 5 minutes (Wang, Jiang et al. 1995). However, HIF-2α protein has been detected in normoxic conditions in a number of endothelial, fibroblast-like and epithelial cell lines to a higher degree than that of HIF-1α (Wiesener, Turley et al. 1998). In addition, HIF-2α is increased to a greater extent than HIF-1α following exposure to mild hypoxia (5% O_2) (Wiesener, Turley et al. 1998) (Holmquist-Mengelbier, Fredlund et al. 2006). The degradation of the HIF-α protein and its stabilisation under hypoxia has been the focus of much research in the last ten years. It has now been proposed that HIF-1α stabilisation in hypoxia (1% O_2) is an acute response to hypoxia, which is diminished under prolonged periods of low oxygen exposure (reviewed in (Lofstedt, Fredlund et al. 2007). Conversely, HIF-2α protein levels are increased under mild hypoxia (5% O_2) and continue to increase with time and may therefore play important roles under prolonged exposure to low oxygen.

There are two transcriptional activation domains in the C-terminal half of both HIF-1α and HIF-2α; the N-terminal activation domain (NAD) and the C-terminal activation domain (CAD) (Jiang, Zheng et al. 1997; Pugh, O'Rourke et al. 1997; Ema, Hirota et al. 1999; O'Rourke, Tian et al. 1999; Huang and Bunn 2003). It is now understood that under normoxic conditions the α subunit is hydroxylated at conserved proline

residues (HIF-1α Pro^{564} and Pro^{402}) (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Masson, Willam et al. 2001) contained within a unique oxygen-dependent degradation (ODD) domain of the α subunit that critically controls protein stability (Huang, Gu et al. 1998; Srinivas, Zhang et al. 1999; Huang and Bunn 2003; Chan, Sutphin et al. 2005) (Figure 1-8). The sites of proline hydroxylation reside within an LXXLAP motif that is strongly conserved between the two hydroxylation sites of HIF-1α and HIF-2α and between HIF isoforms from different species (Huang, Zhao et al. 2002). The enzymes promoting the hydroxylation of these residues are prolyl-4-hydroxylases (PHD; members of the superfamily of 2-oxogluterate-dependent and related dioxygenases) of which there are three isoforms, PHD1, PHD2, and PHD3 (also called EGLN2, EGLN1, and EGLN3 or HIF prolyl hydroxylase 3 (HPH3), HPH2 and HPH1, respectively) (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Jaakkola, Mole et al. 2001). They have an absolute requirement for Fe (II) as a cofactor and dioxygen as a co-substrate, but do not require ATP or NAD (P) (Jaakkola, Mole et al. 2001). The requirement of the PHD for molecular oxygen means that this reaction can act as an “oxygen sensor” (Mole, Pugh et al. 2002). Recently, a unique proline 4-hydroxylase-related protein (PH-4) has been identified which also suppresses HIF activity in a Pro^{402} and Pro^{564} dependent manner (Oehme, Ellinghaus et al. 2002).

Once the proline residues of HIF-1α are hydroxylated, the von Hippel Lindau (VHL) tumour suppressor gene product (pVHL) is able to bind (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Mole, Pugh et al. 2002). pVHL is then able to regulate HIF-1α proteolysis (Maxwell, Wiesener et al. 1999) by acting as the recognition component of an E3 ubiquitin protein ligase complex (Lisztwan, Imbert et al. 1999; Cockman, Masson et al. 2000; Ohh, Park et al. 2000) to mediate polyubiquitination

and proteosomal degradation of HIF-1α under normoxic conditions (Salceda and Caro 1997; Huang, Gu et al. 1998; Kallio, Wilson et al. 1999; Tanimoto, Makino et al. 2000) (Figure 1-8). Recently, pVHL-interacting deubiquitinating enzyme 2 (VDU2) has been shown to deubiquitinate and stabilise HIF-1α thereby providing another level of control for HIF-1α stabilisation (Li, Wang et al. 2005). In addition, pVHL has also been shown to be regulated by pH. Hypoxic induction or normoxic acidosis can neutralise the function of VHL by triggering its nucleolar sequestration, VHL is then confined to the nucleolus until neutral pH conditions are reinstated (Mekhail, Gunaratnam et al. 2004).

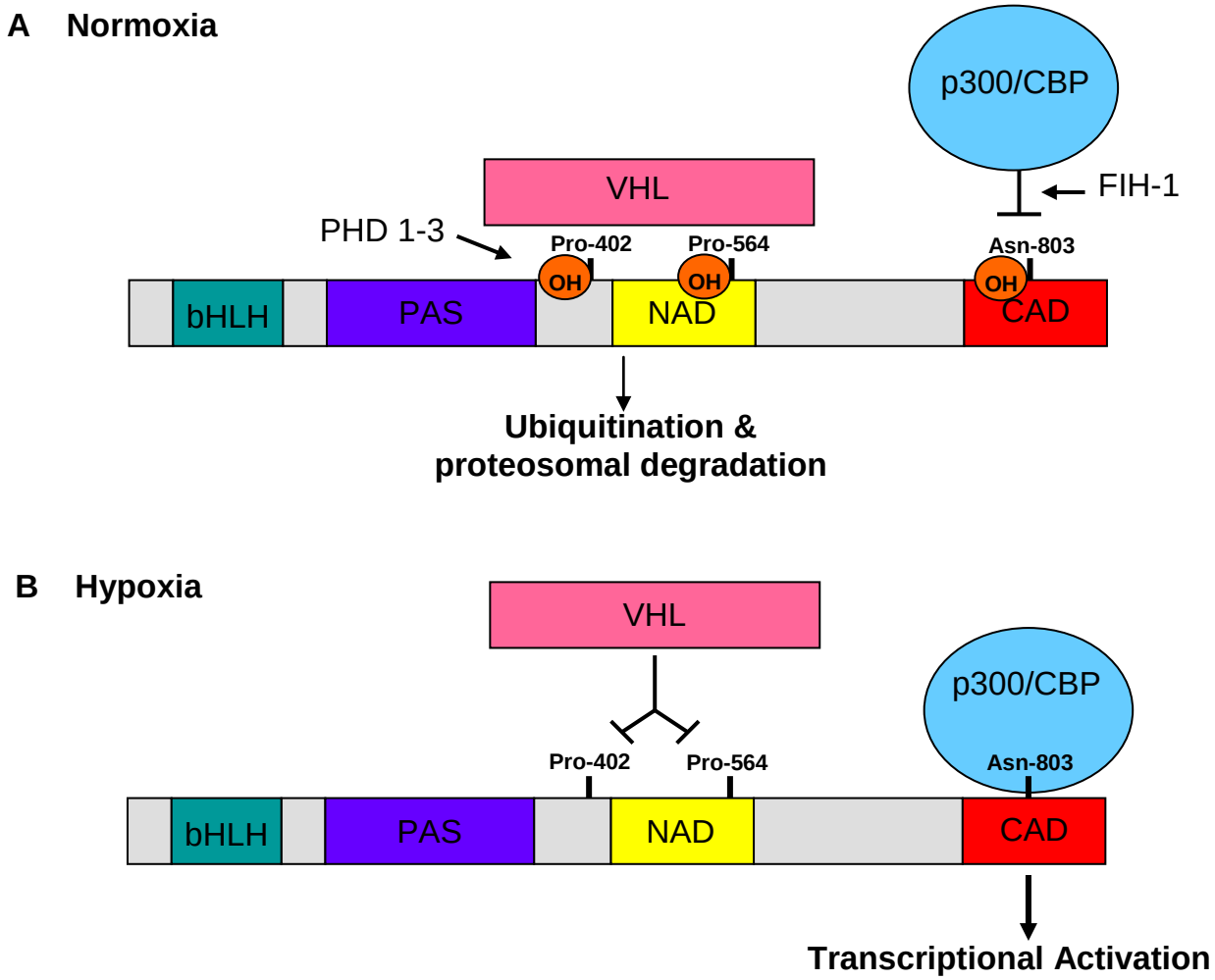


Figure 1-8 Schematic representation of HIF-1α stabilisation and activation under hypoxia
(**A**) When oxygen is available to the cell, HIF-1α is hydroxylated at conserved proline residues (Pro402 and Pro564) by prolyl-4 hydroxylases (PHDs). This enables VHL to bind and target HIF-1α for degradation. In addition, factor inhibiting HIF (FIH)-1 hydroxylates HIF-1α at Asn803 and prevents p300/CBP from HIF-1 transactivation. (**B**) In a low oxygen environment, the alpha subunit is unable to be hydroxylated, this stabilizes the protein and allows transcriptional activation.

The α subunits have also been found to be regulated at the mRNA level. Recently it has been shown that *Hif-3α*, but not *Hif-1α* or *Hif-2α*, mRNA levels increased after 2 hours of hypoxic exposure but no change was seen within 30 minutes (Heidbreder, Frohlich et al. 2003). In contrast, a previous study had shown that *Hif-1α* mRNA was able to be induced within 30 min of hypoxia treatment but returns to baseline after 4 hours (Wiener, Booth et al. 1996). Similarly, Wang et al. showed that hypoxia induces the expression of *Hif-1* mRNA but this is diminished by longer periods of hypoxia (Wang, Jiang et al. 1995) (Uchida, Rossignol et al. 2004). This suggests that *Hif-1α* mRNA is transiently increased. Consistent with these, another study also found that 4 hours of hypoxia had no effect on *Hif-1α* mRNA levels (Wenger, Rolfs et al. 1996) but no earlier time points were examined. In 1999, a naturally occurring antisense transcript of HIF-1α (asHIF-1α) was discovered in human renal cancer (Thrash-Bingham and Tartof 1999). Since then asHIF-1α has also been observed in rodents (Rossignol, de Laplanche et al. 2004). It has been suggested that asHIF-1α exposes AU rich elements in the 3'UTR of *Hif-1α* mRNA which decreases the stability of *Hif-1α* mRNA and finally decreases HIF-1α protein expression (Rossignol, Vache et al. 2002). Conversely, *Hif-2α* mRNA is not destabilised by asHIF-1α due to the lack of AU rich elements (Uchida, Rossignol et al. 2004). In fact, *Hif-2α* transcription can increase in hypoxia due to HIF-2α increasing its own promoter activity (Sato, Tanaka et al. 2002). Since asHIF-1α contains a putative HRE it is assumed that both HIF-1 and HIF-2 are involved in the regulation of asHIF-1α expression thus creating a negative feedback loop of HIF regulation under prolonged hypoxia (Rossignol, Vache et al. 2002; Uchida, Rossignol et al. 2004).

1.9.2 Regulation and Activity of PHDs

The PHD isoforms have formed the basis of much research in recent years and it is now known that they themselves are tightly controlled and form yet another area of regulation in the HIF system. PHD1 has been found to be exclusively present in the nucleus, the majority of PHD2 is mainly located in the cytoplasm whereas PHD3 is distributed across the cytoplasm and the nucleus (Metzen, Berchner-Pfannschmidt et al. 2003). The expression of PHD2 and PHD3, but not PHD1, is hypoxia-inducible (Metzen, Berchner-Pfannschmidt et al. 2003; Appelhoff, Tian et al. 2004) and HIF-1 dependent (del Peso, Castellanos et al. 2003; Marxsen, Stengel et al. 2004), thereby supporting a role for these enzymes in a negative feedback pathway of HIF-α degradation. In addition, it has recently been shown that PHD1 and PHD3 are themselves targets for proteosomal degradation (Nakayama, Frew et al. 2004).

Current evidence suggests that, among the PHD isoforms, PHD2 has the highest activity toward the HIF-1α peptide under normal circumstances (Huang, Zhao et al. 2002; Berra, Benizri et al. 2003; Appelhoff, Tian et al. 2004). However, the contribution of each PHD enzyme seems to be strongly dependent on the cell type and the abundance of the enzyme as, under certain conditions, PHD3 can contribute to HIF regulation at an equal if not greater extent than PHD2 (Appelhoff, Tian et al. 2004).

1.9.3 Regulation of HIF Activity

The second means of HIF inhibition during normoxia involves the hydroxylation of a conserved asparagine residue in the CAD (Lando, Peet et al. 2002) by factor inhibiting HIF-1 (FIH-1), an asparaginyl hydroxylase enzyme (Mahon, Hirota et al. 2001; Hewitson, McNeill et al. 2002; Lando, Peet et al. 2002) (Figure 1-8). FIH

interacts with HIF-1α as well as with VHL and both FIH-1 and VHL inhibit HIF-1α transactivation domain function (Mahon, Hirota et al. 2001). VHL and HIF-1α interact with FIH-1 at distinct sites, with the VHL-binding site located N-terminal to the HIF-1α binding site (Mahon, Hirota et al. 2001). Therefore, the hypoxic regulation of HIF requires firstly the stabilisation of HIF-1α subunit and secondly the activation of the CAD (Minet, Michel et al. 2000). Like other known hydroxylase enzymes, FIH-1 is an Fe(II)-dependent enzyme that uses molecular oxygen to modify its substrate (Lando, Peet et al. 2002). Asparagine 851 in HIF-2α is highly conserved across species, as is the equivalent Asparagine 803 in HIF-1α (Lando, Peet et al. 2002). Through FIH-1 hydroxylation the CAD is silenced and therefore HIF-1α and HIF-2α are unable to interact with the transcriptional co-activators p300/CBP (Lando, Peet et al. 2002). During hypoxia the non-hydroxylated CAD is free to bind to p300/CBP through the interaction with the CH1 domain (Arany, Huang et al. 1996; Dames, Martinez-Yamout et al. 2002; Freedman, Sun et al. 2002; Lando, Peet et al. 2002).

Another negative regulator of hypoxia-inducible gene expression has also been identified. Inhibitory PAS domain Protein (IPAS) acts as a dominant negative regulator of HIF-1α and HIF-2α transcriptional activity (Makino, Cao et al. 2001). IPAS dimerises with HIF-α subunits and prevents the interaction between HIF and the HRE of its target genes (Makino, Cao et al. 2001). Later, it was found that IPAS is an alternative slice variant of HIF-3α and is hypoxia inducible, thus providing another negative feedback pathway in the regulation of HIF activity (Makino, Kanopka et al. 2002). Similarly, HIF-3α itself can suppress HIF-mediated gene transcription in the human kidney (Hara, Hamada et al. 2001).

1.9.4 Cytoplasmic shuttling and HIF regulation

Recent research has been focused somewhat on the cytoplasmic shuttling of HIF-1α and factors involved in its regulation. HIF-1α accumulation in the nucleus under hypoxic conditions is independent of ARNT but the formation of the HIF complex occurs in the nucleus and ARNT is required to retain nuclear HIF-1α (Chilov, Camenisch et al. 1999). Importantly, nuclear translocation is not necessary for HIF-1α stabilisation, as both nuclear and cytoplasmic proteosomes are fully competent for HIF-1α degradation in an oxygen-dependent manner (Berra, Roux et al. 2001). It has therefore been deduced that hydroxylation of HIF-1α and subsequent ubiquitination must take place in both cellular compartments (Berra, Roux et al. 2001). In contrast, a subsequent study found that upon reoxygenation of cells, HIF-1α binds to VHL and is ubiquitinated in the nucleus before being transported by the ubiquitin-ligase complex to the cytoplasm for proteosomal degradation (Groulx and Lee 2002). Also, prolyl hydroxylation of the ODD domain is necessary for both ubiquitination and nuclear export. However, this paper only investigated reoxygenated cells, and hence degradation of newly synthesised HIF-1α in both compartments in oxygenated cells cannot be ruled out.

1.9.5 Non-hypoxic induction of HIF-α

Recently, it has been discovered that HIF-1α protein can also be induced by factors other than hypoxia, including hormones and growth factors. HIF-1α can be regulated by a form of progesterone (P_4) in the mouse uterus and estrogen has the ability to regulate HIF-2α (Daikoku, Matsumoto et al. 2003). Similarly, exposure of colon cancer cells to prostaglandin E_2 (PGE_2) induces the expression of VEGF via increased HIF-1α (Fukuda, Kelly et al. 2003). HIF-1α levels in vascular smooth

muscle cells are strongly increased in normal oxygen conditions when cells are stimulated with angiotensin II (Ang-II), thrombin, platelet derived growth factor (PDGF) or transforming growth factor (TGF)-β1 (Richard, Berra et al. 2000; Gorlach, Diebold et al. 2001; Page, Robitaille et al. 2002). Other non-hypoxic inducers of HIF-1α include Endothelin 1 (Spinella, Rosano et al. 2002), epidermal growth factor (EGF) (Jiang, Jiang et al. 2001), Rho A which regulates actin stress fibers (Hayashi, Sakata et al. 2005) and even cyclical mechanical stretch (Chang, Shyu et al. 2003).

Cytokines have also been shown to regulate HIF in normoxia. For example, IL-1β and TNF-α increased HIF-1 DNA binding in human hepatoma cells (Hellwig-Burgel, Rutkowski et al. 1999). IL-1β increased HIF-1α nuclear protein whereas the effect of TNF-α on HIF-1 DNA binding was suggested to be due to activation of HIF coactivator proteins (Hellwig-Burgel, Rutkowski et al. 1999). Similarly, a more recent study found that IL-1β induces HIF-1α mediated VEGF secretion in normal human trophoblast cells and this may be due to ERK 1/2 activation (Qian, Lin et al. 2004).

In addition, although it is well known that IGF-II is a target gene of HIF-1 (Feldser, Agani et al. 1999), it was recently found that insulin, IGF-I and IGF-II are all able to induce HIF-1α protein expression in various cell lines (Zelzer, Levy et al. 1998; Feldser, Agani et al. 1999; Jiang, Jiang et al. 2001; Chavez and LaManna 2002) but the magnitude of this response *in vitro* is far less than that caused by hypoxia. Similarly, it has also been reported that insulin induces HIF-1α subunit accumulation, HIF-1α activation and VEGF expression, in both whole cell lysates and in nuclear extracts, in a time and concentration dependent manner (Treins, Giorgetti-Peraldi et al. 2002). Similarly, a recent study found that both insulin and 2-deoxy-D-glucose resulted in a widespread increase in HIF-3α mRNA and protein (Heidbreder et al.

2007). IGF-I can also induce VEGF expression in human osteoblast-like cells through transcriptional activation involving the HIF-2α/ARNT complex (Akeno, Robins et al. 2002).

Research has also focused on the mechanisms by which insulin, IGF-I and IGF-II induce HIF-α. At present the receptor that mediates this action remains elusive and may be dependent on the cell type or the growth factor tested. In mouse embryonic fibroblasts it was found that the IGFs and insulin are able to use receptors other than, or in addition to, the IGF1R to induce HIF-1α expression (Feldser, Agani et al. 1999). More recently, amnion-derived WISH cells and human colon carcinoma cells increased HIF-1α protein and *Vegf* mRNA in response to IGF-I, and its activation of MAPK and PI-3K, suggesting that this was mediated by the IGF1R (Fukuda, Hirota et al. 2002; Kawano, Nakamura et al. 2004). Interestingly, infusion of an IGF1R antagonist abrogated HIF-1α accumulation after cerebral ischemia in rats (Chavez and LaManna 2002). Conversely, in hepatic cells, insulin was shown to act through a receptor other than an IGF receptor (Zelzer, Levy et al. 1998). Once bound to a receptor, the PI3 and MAPK pathways are required to transduce the signal and increase the expression of HIF-1α (Jiang, Jiang et al. 2001; Fukuda, Hirota et al. 2002; Treins, Giorgetti-Peraldi et al. 2002; Burroughs, Oh et al. 2003).

The increase in HIF-1α by IGFs is not seen at the mRNA level but at the protein level, and unlike in hypoxia, this does not appear to involve increased stabilisation of the protein but rather an increase in translation of existing mRNA (Fukuda, Hirota et al. 2002). In contrast, it has been suggested that growth factors such as IGF-I could target PHD2 (Berra, Benizri et al. 2003). To add yet another layer of complexity to the system, it was recently found in a human keratinocyte cell line, HaCaT, that IGF-II

induces the expression of MDM2 through the MAP kinase pathway and this, in turn, decreases levels of p53 followed by increased HIF-1α (Kwon, Kwon et al. 2004).

1.10 Hypoxia Inducible Factors in Pregnancy

Both HIF-1 and HIF-2 are essential for embryonic and placental development (Huang and Bunn 2003). Both HIF-1α and HIF-2α protein are constitutively expressed in the human placenta, their abundance decreasing significantly with gestational age (Caniggia, Mostachfi et al. 2000; Rajakumar and Conrad 2000; Caniggia and Winter 2002). In contrast, the levels of *Hif-1α* mRNA in placenta do not change across gestation whilst *Hif-2α* mRNA abundance increases with increasing gestational age (Rajakumar and Conrad 2000). In first trimester human placenta, HIF-1α and -2α have been co-localised in the STB, villous CTB and fetoplacental vascular endothelium (Rajakumar and Conrad 2000). Another study found similar results with regards to HIF-2α, reporting that in the first trimester it was localised to the cytoplasm of CTBs in cell columns, with much more intense nuclear localisation in CTBs in the distal regions of the cell column and in the invasive CTBs (Genbacev, Krtolica et al. 2001). However, only low levels of HIF-1α localisation in CTBs were reported (Genbacev, Krtolica et al. 2001). Interestingly, in first trimester human placenta, VHL was also localised at sites of column initiation and in the proximal column region (Genbacev, Krtolica et al. 2001). Interestingly, the expression of HIF-1α in preeclamptic trophoblasts is similar to the expression seen in trophoblasts prior to their exposure to increased oxygen (Caniggia and Winter 2002). HIF-1α and HIF-2α are over-expressed in the villous placenta of women with preeclampsia compared to normal term pregnancies (Rajakumar, Whitelock et al. 2001) and oxygen dependent downregulation of HIF-1α and -2α protein is impaired (Rajakumar, Doty et al. 2003).

In vitro studies have yielded contradictory results. In one study, human placental villous explants from 5-8 weeks gestation, cultured at 3% oxygen demonstrated significantly greater mRNA expression of *Hif-1α* when compared with explants cultured at 20% (Caniggia, Mostachfi et al. 2000). In contrast, first trimester or term villous explants that were exposed to hypoxia showed increases in HIF-1α and HIF-2α protein expression, with no change in their mRNA levels, and increased HIF-1 DNA binding activity (Rajakumar and Conrad 2000). However, another group found that in cultured 6-8 week placenta it was HIF-2α protein that dramaticaly increased during hypoxia, while HIF-1α levels were the same in cells cultured under hypoxic and normoxic conditions (Genbacev, Krtolica et al. 2001).

HIFs have also been localised in the early murine placenta. *Hif* mRNA expression was low to undetectable on day 1 of pregnancy, increasing by day 4 where *Hif-1α* was distinctly expressed in the luminal epithelium and *Hif-2α* was localised to the stroma (Daikoku, Matsumoto et al. 2003). On day 5 of pregnancy both the luminal epithelium and stroma exhibited *Hif-1α* mRNA expression, whereas *Hif-2α* mRNA expression was restricted to only stromal cells surrounding the blastocyst (Daikoku, Matsumoto et al. 2003). In contrast, the mRNA expression of *Hif-3α* was very low without any cell-specific localisation. On day 8, *Hif-1α* mRNA expression showed further increases in the decidual bed, but the most robust mRNA expression was noted for *Hif-2α* (Daikoku, Matsumoto et al. 2003). During mid pregnancy (days 9.5-10.5), expression of *Hif-1α* mRNA was detected throughout the placenta with the highest expression at the fetomaternal border (Schaffer, Vogel et al. 2005). In contrast, *Hif-2α* mRNA was predominantly localised to the maternal decidua at this time. A previous study had found similar expression of *Hif-2α* mRNA in the decidual cells of the day 9.5 murine placenta (Jain, Maltepe et al. 1998). In mice, ARNT is

expressed in the nuclei of the maternal decidual cells, in embryonic trophoblasts, and in the single layer of trophoblast giant cells which surrounds the embryo and lies between the maternal and embryonic placental regions (Kozak, Abbott et al. 1997). VHL was detected by immunohistochemistry in the labyrinthine trophoblasts, the allantoic mesoderm, and some placental embryonic endothelium in wild type animals (Gnarra, Ward et al. 1997).

Very little research has been done on the localisation of HIF-1α protein in mice. On day 8.5 there was no HIF-1α protein detected in the EPC (Schaffer, Vogel et al. 2005). Similarly, HIF-1α protein was barely detectable in the placenta during mid pregnancy and was mainly confined to TGCs at the periphery of the placenta (Schaffer, Vogel et al. 2005). No studies have been done to investigate the expression of the HIFs in the second half of murine pregnancy.

Interestingly, in placentas from hypoxia treated mice in mid-pregnancy there was a significant increase in *Hif-2α*, but not *Hif-1α*, mRNA abundance in the decidua on day (Schaffer, Vogel et al. 2005). However, HIF-1α protein was increased in the TGCs and the junctional zone after mice were subjected to hypoxia *in vivo* (Schaffer, Vogel et al. 2005). Rat Rcho-1 cells cultured in 5% oxygen had increased expression of HIF-1α protein compared to those cultured under 20% (Hayashi, Sakata et al. 2004).

1.10.1 HIF System Gene Ablation Studies

Hif-1α deficient embryos display development arrest starting at day 8 followed by gross morphological abnormalities, defects in vascularisation and embryonic lethality by day 11 (Iyer, Kotch et al. 1998; Ryan, Lo et al. 1998; Cowden Dahl, Fryer et al. 2005). Similarly, *Arnt* -/- embryos die between day 9.5 and 10.5 due to impaired

vascularisation of the embryonic component of the placenta (Kozak, Abbott et al. 1997). More than half of *Hif-2α* (*Epas-1*) knockout embryos die from vascular defects, typically in the yolk sac before day 13.5 (Peng, Zhang et al. 2000). Those that were born alive survived for several weeks. A similar study showed that half of all *Hif-2α* knockout embryos die at day 13.5 due to cardiac failure and those that do go to term die within 2-3 hours due to severe respiratory failure (Compernolle, Brusselmans et al. 2002). In these mice, absence of VEGF *in utero* impaired lung maturation and resulted in respiratory distress syndrome at birth due to insufficient surfactant production.

Hif1α -/- mouse placentas exhibit impaired chorioallantoic fusion and vascularisation of the chorion (Cowden Dahl, Fryer et al. 2005). In contrast, *Hif-2α* -/- placentas have normal numbers of fetal blood vessels and proper invasion into maternal tissues (Cowden Dahl, Fryer et al. 2005). Defects in placentation were further exacerbated in *Hif1α* -/- *Hif2α* -/- mice which had no allantoic fetal blood vessels, hypocellularity of multiple trophoblast cell layers and a 17% decrease in placental invasion compared to wild type placentas (Cowden Dahl, Fryer et al. 2005). This is almost identical to *Arnt* -/- placentas which also have no fetal blood vessels and a decrease in placental invasion (Adelman, Gertsenstein et al. 2000). These poorly invasive placentas from the *Arnt* -/- and *Hif1α* -/- *Hif2α* -/- mice also have decreased spongiotrophoblast cell numbers and expanded TGC numbers (Adelman, Gertsenstein et al. 2000; Cowden Dahl, Fryer et al. 2005). Therefore, it appears that HIF-1 and HIF-2, in cooperation with ARNT, regulate placental vascularisation and invasion, as well as trophoblast differentiation.

Surprisingly, *Vhl*-deficient mice develop placental lesions at 9.5 to 10.5 days of gestation and die *in utero* between days 10.5 to 12.5 due to the absence of placental and embryonic vasculogenesis and subsequent haemorrhage and necrosis (Gnarra, Ward et al. 1997). *Vhl* -/- embryos had a lack of fetal endothelium and fetal blood vessels in the placental labyrinth (Gnarra, Ward et al. 1997). The trophoblasts appeared normal and viable until about day 9.5, but failed to progress and develop syncytiotrophoblast (Gnarra, Ward et al. 1997). VEGF protein levels were greatly reduced in the labyrinthine trophoblasts of *Vhl* deficient placentas, implying that the decreased VEGF levels seen in *Vhl* deficient embryos may play a contributory role in embryonic lethality (Gnarra, Ward et al. 1997).

Phd2 -/- embryos die on days 12.5 - 14.5, preceded by severe placental and heart defects, whereas *Phd1* -/- or *Phd3* -/- mice are apparently normal (Takeda, Ho et al. 2006). *Phd2* -/- mice display global increases in HIF-α protein in the placenta and the embryo proper. In addition, placentas from *Phd2* -/- mice have reduced labyrinthine branching, increased spongiotrophoblasts, and abnormal distribution of TGCs (Takeda, Ho et al. 2006). They also have increased expression of spongiotrophoblast marker *Mash2* and decreases in the labyrinthine markers *Tfeb* and *Gcm1*.

1.11 Conclusion, Hypotheses and Aims

IGF-II is a key regulator of blastocyst implantation and placentation but it is important to remember that it does not act alone. As discussed here, IGF-II can interact with a variety of other factors including the uPA system, oxygen and HIFs to mediate its effects.

Firstly, we aim to elucidate the effects of a combination treatment of IGF-II, uPA and plasminogen on blastocyst development, subsequent implantation and fetal and placental growth. We hypothesise that this treatment will overcome the adverse effects of *in vitro* embryo culture to improve implantation rates in mice.

Secondly, we will provide an account of early murine placental development, including a description of trophoblast migration and invasion and the remodeling of the maternal vasculature. To date most of the research has focused on the later stages of placental development. However, we aim to look early in pregnancy when trophoblasts are at their most invasive, when essential decidual remodelling and angiogenesis are maximal and when placental morphogenesis is initiated. In addition, we aim to determine the localisation of IGF-II and its receptors in early murine implantation sites in order to better understand their possible roles in early mouse placentation. We hypothesise that IGF-II and its receptors will be co-localised to trophoblast cells supporting their role in trophoblast migration and function.

Finally, we aim to investigate the interactions between IGF-II, oxygen and HIFs in regulating trophoblast migration, differentiation and function. More specifically, we hypothesise that IGF-II, in a low oxygen environment, interacts with HIFs to promote trophoblast migration, differentiation and altered trophoblast gene expression.

Chapter 2
IGF-II, uPA and plasminogen, in combination, improves embryo development and implantation *in vitro*

2.1 Introduction

Embryo culture is a vital component of assisted reproductive technologies (ART). However, the *in vitro* environment is by no means optimal. In a normal pregnancy, the developing embryo is exposed to an array of growth factors and other molecules, which are either produced by the reproductive tract or by the embryo itself. The *in vitro* culture media systems used today only partially mimic the physiological situation and currently there are none that contain any of the growth factors to which the pre-implantation embryo is normally exposed. This poor *in vitro* environment may contribute to the high rate of implantation failure seen in ART following embryo transfer.

In recent years, several growth factors and other molecules have been identified that can improve blastocyst development. Insulin-like growth factor (IGF-II) has been one growth factor, in particular, that has been added to embryo culture media with the aim of improving blastocyst outcome. Normally, in mouse embryos *Igf2* mRNA is first expressed at the 2-cell stage of development (Rappolee, Sturm et al. 1992; Schultz, Hogan et al. 1992; Schultz, Hahnel et al. 1993) and IGF-II protein has been detected in all cells of the mouse blastocyst (Rappolee, Sturm et al. 1992). However, expression of *Igf2* mRNA is delayed and IGF-II protein reduced following *in vitro* fertilisation (IVF) and culture of mouse embryos (Stojanov, Alechna et al. 1999). Interestingly, mouse embryos cultured with antisense IGF-II oligonucleotides have delayed blastocyst formation, and 20% fewer cells (Rappolee, Sturm et al. 1992), whilst addition of IGF-II to mouse embryo culture media improves development to the blastocyst stage and increases the proportion of blastocysts hatching (Harvey and Kaye 1992; Harvey and Kaye 1992; O'Neill 1997; Kurzawa, Glabowski et al. 2001; Lin, Yen et al. 2003; Pantaleon, Jericho et al. 2003). Exogenous IGF-II also

increases total blastocyst, inner cell mass (ICM) and trophectoderm (TE) cell numbers and increases glucose uptake by the mouse blastocyst (Harvey and Kaye 1992; O'Neill 1997; Pantaleon, Jericho et al. 2003).

Successful implantation of the embryo into the uterine stroma is a highly controlled process of tissue invasion and involves the local production and activation of ECM-degrading proteinases by the trophectoderm. It seems then, that a combination of both growth factors and ECM degrading enzymes may be required to reverse the detrimental effects of *in vitro* culture on blastocyst development and embryo implantation.

Urokinase plasminogen activator (uPA), uPA receptor (uPAR) and plasminogen are known to play important roles in cellular invasion. Binding of the inactive pro-enzyme, pro-uPA, to the uPAR cleaves the protein to form active uPA. Cell bound uPA can then cleave the inactive plasminogen to the proteinase plasmin (Kjoller, Kanse et al. 1997) which can then go on to activate MMPs and directly degrade certain ECM components and promote invasion. *In vivo*, expression of uPA and uPAR in the mouse embryo is increased upon blastocyst formation, just prior to implantation (Sappino, Huarte et al. 1989; Harvey, Leco et al. 1995). uPA is mainly localised to the trophectoderm (Aflalo, Sod-Moriah et al. 2005; Aflalo, Sod-Moriah et al. 2007) and its activity is increased both *in vivo* and *in vitro* in rat blastocysts (Zhang, Kidder et al. 1994; Aflalo, Sod-Moriah et al. 2004). However, as for IGF-II, *in vitro* culture reduces uPA protein expression and activity in rat embryos (Aflalo, Sod-Moriah et al. 2004; Aflalo, Sod-Moriah et al. 2005). uPA is also expressed in trophoblast outgrowths from blastocysts *in vitro*, and is expressed by the trophoblast giant cells (TGCs) and in the ectoplacental cone (EPC) just after implantation (day 5.5 and 6.5) in the mouse

(Sappino, Huarte et al. 1989; Teesalu, Blasi et al. 1996). uPA has been shown to be important for blastocyst attachment and implantation. T^{w73} mutant embryos which have reduced levels of plasminogen activators do not implant (Axelrod 1985) and inhibition of uPA has been shown to decrease the extent of trophoblast outgrowth and attachment *in vitro* (Kubo, Spindle et al. 1981).

In addition, plasmin and plasminogen have been shown to improve blastocyst development and hatching rates, and increase attachment and subsequent outgrowth (Menino and O'Claray 1986; Menino, Dyk et al. 1989). This may be due to the ability of plasmin to proteolytically degrade the zona pellucida, as seen in bovine embryos (Cannon and Menino 1998).

Recently, a novel interaction between IGF-II and the uPA system has been identified. This interaction may be important for blastocyst implantation *in vivo* and may also therefore be an ideal candidate for a new combination media formulation for *in vitro* embryo culture. IGF2R and uPAR have been found to interact with each other, at sites different from those used for binding IGF-II or uPA, respectively, to form part of a large plasma membrane complex (Nykjaer, Christensen et al. 1998; Godar, Horejsi et al. 1999; Kreiling, Byrd et al. 2003; Olson, Yammani et al. 2004). IGF-II induced cell migration has been shown to be dependent on uPAR, uPA and plasmin activity in a rhabdomyosarcoma cell line (Gallicchio, Kaun et al. 2003). In addition, IGF2R also binds to plasminogen and controls the conversion of plasminogen to plasmin (Godar, Horejsi et al. 1999; Olson, Yammani et al. 2004). Therefore, it has been hypothesised that binding of IGF-II to the IGF2R may increase IGF2R/uPAR interactions and/or increase uPA binding to uPAR, resulting in increased plasminogen conversion to plasmin at the cell surface to increase cell migration (Gallicchio, Kaun et al. 2003).

We hypothesised that this novel interaction might also be necessary for blastocyst hatching and subsequent implantation and placentation. We aimed to determine whether IGF-II, uPA and plasminogen, in combination, would ameliorate the detrimental effects of *in vitro* culture by improving blastocyst development and hatching, as well as enhancing subsequent implantation after embryo transfer.

2.2 Methods

Ethics approval for this study was obtained from the University of Adelaide's animal ethics committee. All mice were obtained from the University of Adelaide Central Animal House and were housed in clean conventional conditions on a cycle of 12 hours light, 12 hours dark and received food and water *ad libitum*.

2.2.1 Embryo Collection and Culture

C57Bl/6 female mice (21-25 days old) were injected subcutaneously at 1600 h with 5IU of equine chorionic gonadotrophin (eCG; Folligon, Intervet, Bendigo, VIC, Australia) followed by a second injection 48h later of 5IU human chorionic gonadotrophin (hCG; Chorulon, Intervet). Immediately following the second injection, females were placed with BalbC males overnight for mating. Twenty-three hours post hCG, females were sacrificed by cervical dislocation and 1-cell embryos were collected from the oviducts in BlastAssist® M1 media (MediCult, Jyllinge, Denmark). Embryos were incubated with 1 mg/ml hyaluronidase (Sigma-Aldrich, St. Lois, MO, USA) to remove the cumulus cells and were then washed three times in media before being placed into culture. All embryos were cultured in groups of 10. To determine an optimal culture medium drop size and to determine if IGF-II was having paracrine effects, a preliminary experiment culturing embryos in either 25μl or 50μl drops of media under oil was performed. For each replicate, 10-40 embryos per treatment

were cultured and 10-20 replicates were undertaken. All cultures were performed at 37°C in 6% CO_2, 5%O_2 in nitrogen.

2.2.2 Culture Media and Treatments

The media used for embryo culture was BlastAssist® System M1 and M2 media (MediCult) which is a sequential media system. All embryos were cultured in M1 media overnight for 20 h and assessed for development to the 2-cell stage. Embryos that had reached the 2-cell stage were then cultured in varying concentrations of IGF-II (0 nM, 0.5 nM, 1 nM, 12.5 nM, 25 nM or 50 nM) to determine an optimal dose. In subsequent experiments, to assess the effects of IGF-II, uPA and plasminogen in combination, 2-cell embryos were divided into one of four groups: M1 media alone, media containing 12.5 nM IGF-II (GroPep, Thebarton, SA, Australia), media containing 10 μg/ml urokinase (uPA; Sigma-Aldrich) and 5 μg/ml plasminogen (Sigma-Aldrich), or media containing a combination of the same doses of IGF-II, uPA and plasminogen (All). The concentrations used for uPA and plasminogen have previously been shown to promote trophoblast invasion in a human trophoblast cell line (Roberts, Sferruzzi-Perri et al. 2007). Embryos were washed and then cultured in this media for a further 23 h (see Figure 2-1 for timeline). Cleavage stage embryos were then transferred to M2 media with the appropriate treatment for a further 41 h. All embryo culture dishes were equilibrated to the appropriate temperature and gas mix for at least 4 h before culture.

2.2.3 Assessment of Morphology

Embryo morphology was assessed at 20, 43 and 84 h of culture (Figure 2-1) and embryos were classified as 2–8 cell, morula (fully compacted embryo), blastocyst or hatching blastocyst (clear herniation of, or extrusion through, the zona pellucida by the trophectoderm).

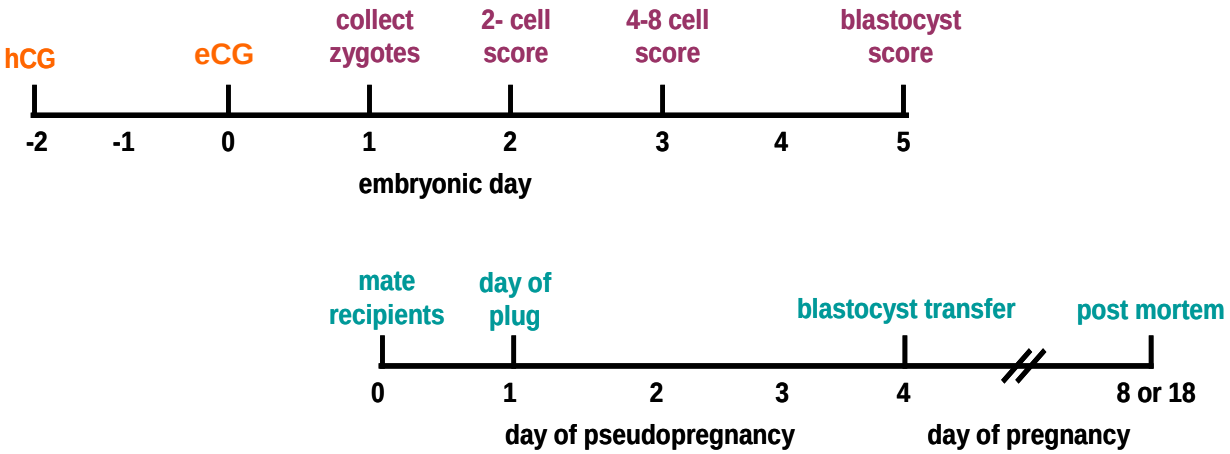


Figure 2-1 Timeline of embryo culture and blastocyst transfer

2.2.4 Embryo Transfers

Naturally ovulating female C57Bl6 mice (8-10 weeks of age) were caged with vasectomised BalbC males and mating was confirmed by the presence of a vaginal plug (day 1 of pseudopregnancy). Blastocysts were transferred to day 4 pseudopregnant recipients at 0800-1000 h, approximately 85-87 h after initiation of culture (Figure 2-1). The surgery was performed under general anaesthesia by i.p. injection of 2% Avertin (2,2,2-Tribromoethanol in 2-methyl-2-butanol, diluted to 2% solution in H_20; 0.015 ml/g body weight). The uterine horns were exposed via a

dorsal incision and five blastocysts were transferred per uterine horn (eg. 10 blastocysts per recipient) using a glass pipette specifically designed for embryo transfer (Swemed AB, Billdal, Sweden). These pipettes have a sharp angle cut edge to allow direct entrance through the uterine wall without the use of a needle. Blastocysts were randomly allocated and transferred to a minimum of 2 recipients on any given day and these were from at least 2 different treatment groups.

2.2.5 Analysis of Implantation and Pregnancy Rates

Recipient mice were killed at midday on day 8 or on day 18 of pregnancy (the day of embryo transfer is defined as day 4 of pseudopregnancy) (Figure 2-1). The number of viable and resorbing implantation sites was recorded.

2.2.6 Statistical Analysis

To analyse differences in embryo development, a univariate analysis of variance (ANOVA) using the day of the replicate as a covariate was performed. Differences in pregnancy and implantation rates were assessed using Chi Square analysis. To determine the effect of treatment on litter size after embryo transfer, a univariate ANOVA with Bonferroni Post Hoc was used. Statistical analyses were carried out using SPSS software (Chicago, IL, USA) except when using Chi Square, in which case GraphPad Prism (San Diego, CA, USA) was used. Differences were considered to be significant if $p < 0.05$.

2.3 Results

2.3.1 Optimisation of Drop Size

To determine an optimal drop size for embryo culture, embryos were cultured in groups of 10 in either 25 μl or 50 μl drops under oil and were cultured in either media alone, media containing 50 nM IGF-II or 100 nM IGF-II. We found that there was no significant difference in blastocyst development in those embryos cultured in 25 μl or 50 μl at any IGF-II concentration (Table 2-1). For all subsequent experiments 50 μl drops were used as we believed that this would dilute out any paracrine effects of endogenous IGF-II and any negative impact of embryonic waste products.

Table 2-1 Effect of different media drop sizes on blastocyst development after 84 h in culture

		< Morula (%)	Morula (%)	Blastocyst (%)	Hatching (%)	Total Blastocyst (%)
25 μl Drop	**Control**	48.7 ± 7.8	9.3 ± 4.9	24.2 ± 7.8	17.8 ± 2.5	42.0 ± 8.4
	50 nM IGF-II	43.1 ± 8.5	7.6 ± 3.2	35.2 ± 3.1	14.1 ± 5.6	49.3 ± 5.8
	100 nM IGF-II	38.0 ± 9.2	12.3 ± 6.2	26.8 ± 13.2	20.0 ± 7.2	49.7 ± 13.2
50 μl Drop	**Control**	34.0 ± 5.3	12.9 ± 5.1	31.6 ± 4.9	18.5 ± 3.1	53.1 ± 8.3
	50 nM IGF-II	31.4 ± 4.2	8.9 ± 3.5	38.4 ± 6.9	21.4 ± 3.7	59.8 ± 5.1
	100 nM IGF-II	33.3 ± 2.1	7.2 ± 4.4	40.1 ± 3.3	19.5 ± 2.0	59.6 ± 4.5

Data are presented as mean ± SEM. % refers to the proportion of 2-cells to reach the morula, blastocyst and hatching blastocyst stages respectively, Total blastocyst is the sum of blastocysts plus hatching blastocysts. N=5-6 replicates per group.

2.3.2 Optimisation of IGF-II concentration

Embryos were cultured in either media alone or increasing concentrations of IGF-II (0.5 nM, 1 nM, 12.5 nM, 25 nM or 50 nM). IGF-II did not alter development of 2-cell embryos to the cleavage stage at any concentration (data not shown). However, IGF-II, only at 12.5 nM, appeared to increase the proportion of 2-cell embryos developing to the blastocyst stage ($p=0.075$) and significantly increased the proportion of blastocysts hatching ($p<0.05$) (Table 2-2).

Table 2-2 Effect of varying concentrations of IGF-II on embryo development after 84 h in culture

	< Morula (%)	Morula (%)	Blastocyst (%)	Hatching (%)	Total Blastocyst (%)
Control	30.8 ± 6.5	25.9 ± 8.1	33.8 ± 5.4	9.4 ± 3.2	43.3 ± 3.9
0.5 nM IGF-II	39.7 ± 2.7	19.3 ± 2.7	29.6 ± 3.5	11.7 ± 3.9	41.0 ± 5.4
1 nM IGF-II	40.5 ± 5.3	14.4 ± 2.5	28.9 ± 4.6	14.7 ± 2.6	45.2 ± 6.0
12.5 nM IGF-II	36.1 ± 2.8	10.9 ± 2.3	37.5 ± 2.9	15.6 ± 2.0 *	53.1 ± 2.8
25 nM IGF-II	39.7 ± 2.9	16.9 ± 2.1	29.5 ± 2.1	13.9 ± 3.0	43.4 ± 2.3
50 nM IGF-II	41.5 ± 5.1	15.1 ± 2.5	31.2 ± 4.3	12.2 ± 3.4	43.4 ± 5.5

* denotes significant difference from controls $p<0.05$. Data are presented as mean ± SEM. % refers to the proportion of 2-cells to reach the morula, blastocyst and hatching blastocyst stages respectively, Total blastocyst is the sum of blastocysts plus hatching blastocysts. N=10 replicates per group.

2.3.3 Embryo Development Rates

Two-cell embryos were cultured in either media alone, media containing IGF-II, media containing uPA and plasminogen or media containing a combination of IGF-II, uPA and plasminogen. Treatments containing uPA and plasminogen separately were not used since preliminary experiments had shown that the effect of these in combination was greater then either alone (data not shown). None of the treatments affected the proportion of 2-cell embryos to reach the cleavage (4-8 cells) stage (Table 2-3).

Table 2-3 Effect of IGF-II, uPA and plasminogen on embryo development to the cleavage stage after 43 h in culture.

	2-cell /frag (%)	3-cell (%)	4-cell (%)	8-cell (%)	Total 4-8 cell (%)
Control	12.4 ± 2.3	6.9 ± 1.2	59 ± 2.3	21 ± 3.0	80.4 ± 2.9
12.5 nM IGF-II	10 .5 ± 1.6	6.2 ± 1.1	53.4 ± 3.7	26.7 ± 3.1	80.1 ± 3.6
10 μg/ml uPA + 5μg/ml plasminogen	8.1 ± 1.9	7.2 ± 0.9	60.0 ± 3.0	24.7 ± 3.0	84.7 ± 2.3
12.5 nM IGF-II, 10 μg/ml uPA & 5 μg/ml plasminogen	8.3 ± 1.5	8.2 ± 1.4	57.8 ± 2.9	26.5 ± 3.5	84.3 ± 2.0

Data are presented as mean ± SEM. % refers to the proportion of 2-cells to reach the morula, blastocyst and hatching blastocyst stages respectively, N= 19-20 replicates per group.

The combination of IGF-II, Plasminogen and uPA appeared to increase development to the blastocyst stage (81%; p=0.059) compared to embryos cultured in media alone (73%) (Table 2-4). Treatment with IGF-II alone, or treatment with uPA and plasminogen without IGF-II, did not alter development to the blastocyst stage. However, the combination of all three factors significantly increased the proportion of blastocysts hatching by 33%, compared to controls (p=0.01) and to IGF-II alone (p<0.001) (Table 2-4). Interestingly, uPA and plasminogen treatment, without IGF-II, did not increase the proportion of blastocysts hatching.

Table 2-4 Effect of IGF-II, uPA and plasminogen in combination, on embryo development after 84 h in culture

	< Morula (%)	Morula (%)	Blastocyst (%)	Hatching (%)	Total Blastocyst (%)
Control	19.1 ± 2.9	7.8 ± 1.7	39.4 ± 2.5	33.7 ± 2.6	73.1 ± 3.3
12.5 nM IGF-II	16.8 ± 2.2	9.5 ± 1.5	40.9 ± 2.9	32.8 ± 2.3	73.7 ± 3.1
10 μg/ml uPA + 5μg/ml plasminogen	12.1 ± 2.5	7.9 ± 1.5	42.2 ± 3.4	37.8 ± 2.4	80.0 ± 3.0
12.5 nM IGF-II, 10 μg/ml uPA & 5 μg/ml plasminogen	12.3 ± 2.3	6.4 ± 1.2	36.7 ± 3.0	44.6 ± 3.0*	81.3 ± 2.6

* Denotes significant difference from embryos cultured in control media (media alone), p<0.01. Data are presented as mean ± SEM. % refers to the proportion of 2-cells to reach the morula, blastocyst and hatching blastocyst stages respectively, Total blastocyst is the sum of blastocysts plus hatching blastocysts. N =19-20 replicates per group.

2.3.4 Effect of embryo culture with IGF-II, uPA and plasminogen on pregnancy outcomes at day 8

After embryo transfer, the combination treatment significantly increased the proportion of mice that were pregnant on day 8 (10 of 11 mice; 91%), compared to controls (6 of 12 mice; 50%, $p<0.0001$), IGF-II alone (6 of 10 mice; 60% $p<0.0001$) and uPA plus plasminogen (8 of 12 mice; 67% $p<0.0001$) (Figure 2-2). In addition, the combination treatment significantly increased the total number of implantations at day 8 per embryos transferred ($p<0.03$) (Figure 2-3). However, in those dams that were pregnant, there was no effect of any of the treatment groups on litter size at day 8 (Figure 2-4) suggesting that the increase in implantation rate was due to the increase in pregnancy rate, rather than an increase in litter size.

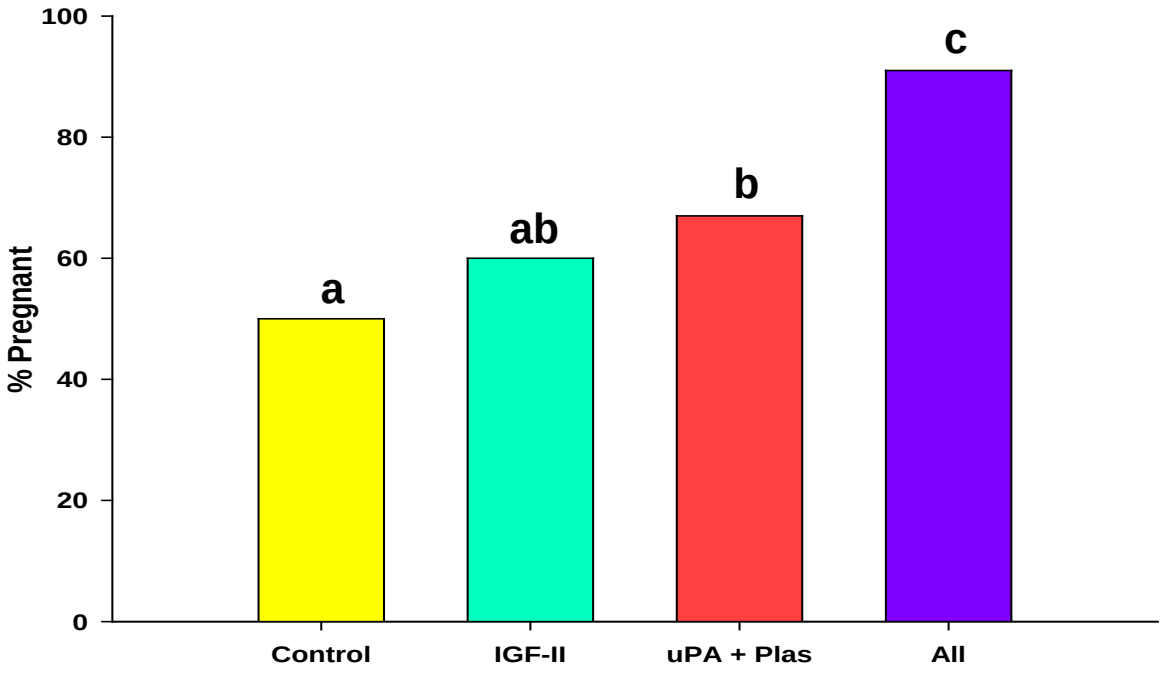


Figure 2-2 Effect of treating embryos with IGF-II, uPA and Plasminogen *in vitro* on pregnancy rate at day 8.
N= 10-12 recipient mothers per group. Different superscripts denote differences between groups, $p<0.0001$.

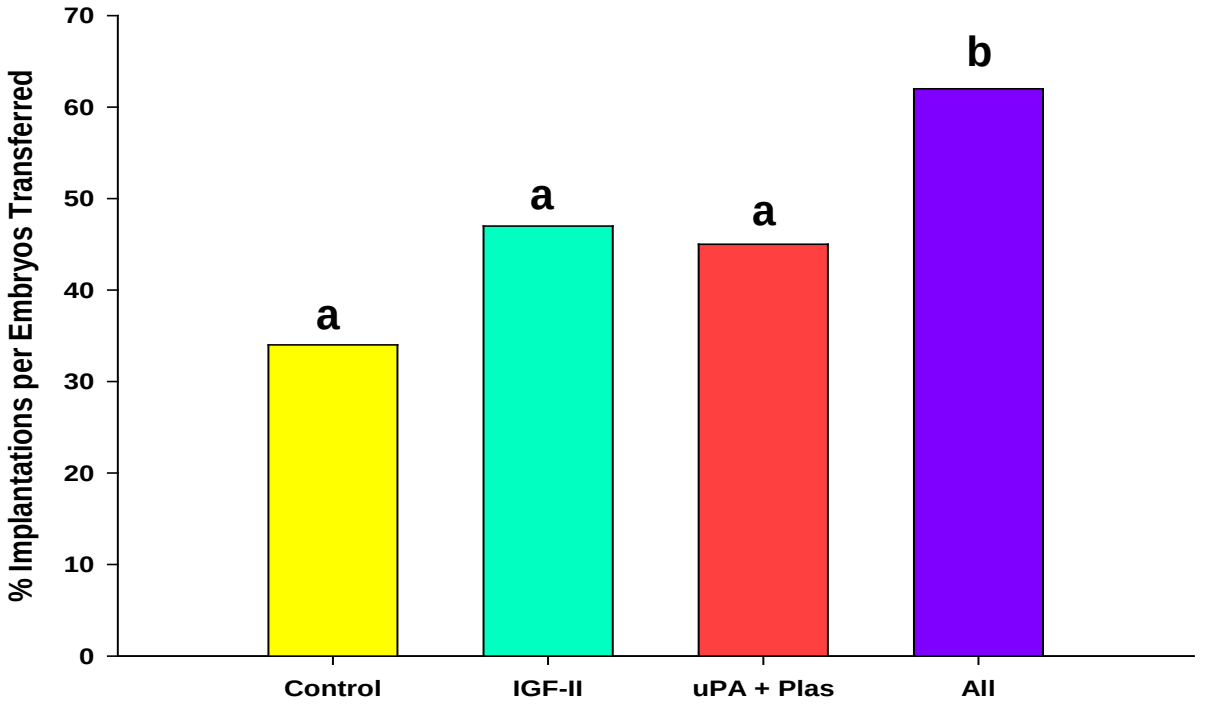


Figure 2-3 Effect of treating embryos with IGF-II, uPA and Plasminogen *in vitro* on implantation rate at day 8.
Data are expressed as total number of implantations per embryos transferred. N= 10-12 recipient mothers per group. Different superscripts denote differences between groups, p<0.0001.

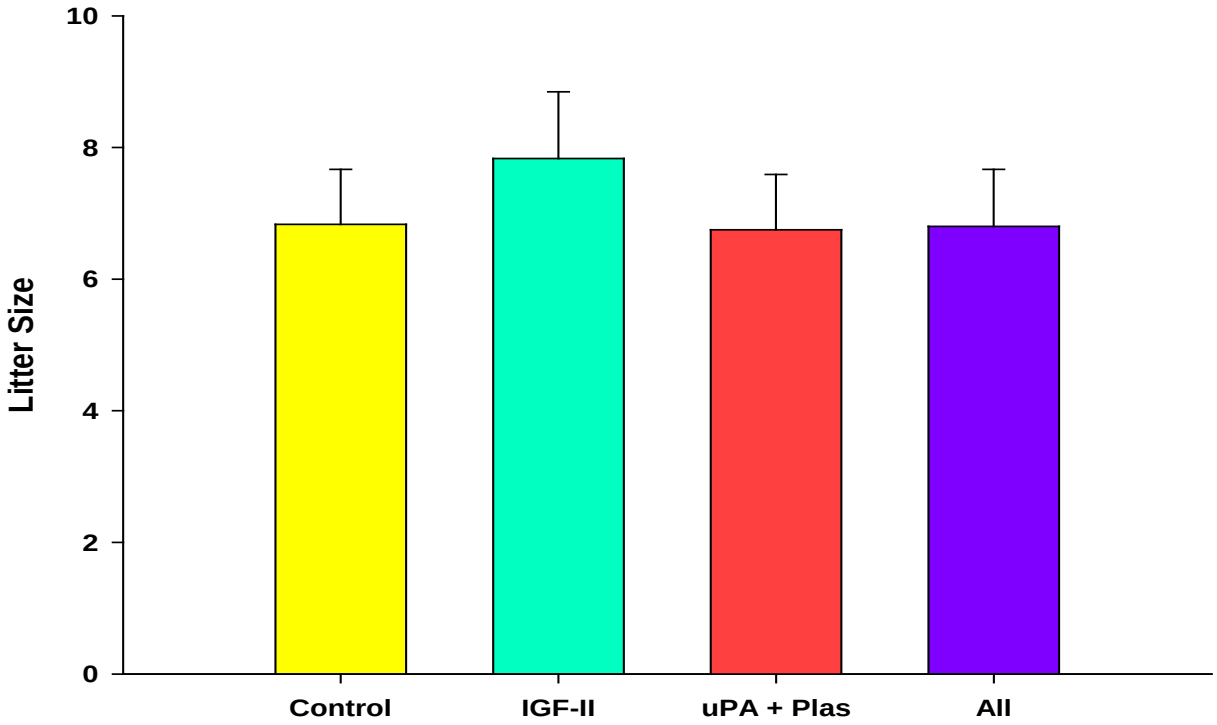


Figure 2-4 Effect of treating embryos with IGF-II, uPA and plasminogen *in vitro* on litter size at Day 8.
Data are presented as mean ± SEM. Values represent mean number of pups per pregnant recipient mother. N= 6-10 pregnant recipient mothers per group.

2.3.5 Effect of Embryo Culture with IGF-II, uPA and Plasminogen on Pregnancy Outcomes at Day 18

By day 18 of pregnancy only one third (10/28) of all recipient mothers had maintained their pregnancy and only 7 of these had viable fetuses. There were a total of 11 viable fetuses and 53 resorptions, suggesting that there was a high rate of fetal loss in mid and late pregnancy. Due to the low success rates in all treatment groups, we were unable to perform statistical analyses on day 18 outcomes.

2.4 Discussion

This study demonstrates that the addition of IGF-II, uPA and plasminogen, in combination, to embryo culture media improves blastocyst hatching and increases subsequent pregnancy and implantation rates following embryo transfer. Previous work has only examined these factors individually and there have been no studies that have determined the effects on implantation following embryo transfer.

In preliminary studies, we demonstrated that a dose of 12.5 nM IGF-II increased the proportion of blastocysts hatching. However, in the subsequent experiment, IGF-II treatment alone had no effect on blastocyst development and it did not alter implantation nor pregnancy rates at day 8. The different effect on embryo development between the first and second experiment may be because the second experiment, incorporating all treatment groups, was undertaken in a new state of the art embryology laboratory whereas the first experiment, examining IGF-II dose response, was performed in less optimised laboratory facilities. In control embryos, 43% developed to blastocysts in the first experiment whereas 73% reached the blastocyst stage in the second experiment. Nevertheless, the initial experiment

provided relevant information as to the optimal dose of IGF-II to be added to the media.

Previously, addition of IGF-II to embryo culture media was shown to increase blastocyst development and the proportion of blastocysts hatching (Harvey and Kaye 1992; Harvey and Kaye 1992; O'Neill 1997; Kurzawa, Glabowski et al. 2001; Lin, Yen et al. 2003; Pantaleon, Jericho et al. 2003). The different effect of IGF-II seen on blastocyst development between this study and those previously, may be because the present study utilised a sequential media system whereas previously only simple, one step embryo culture media have been used. This change to a sequential media system has improved embryo culture and thus the effect of exogenous IGF-II may be somewhat diluted.

The embryo transfer experiments demonstrated that uPA plus plasminogen treatment, without IGF-II, also increased the number of recipients that were pregnant following embryo transfer. Previously, plasminogen treatment alone has been shown to improve blastocyst development and hatching in mouse and sheep embryos (Menino and O'Claray 1986; Menino, Dyk et al. 1989) but no such effects on blastocyst development were seen in the present study with the addition of uPA and plasminogen. This may be due to the high doses of plasminogen used previously, where 14.6 μg/ml plasminogen increased blastocyst development and concentrations of 29.1 μg/ml plasminogen were required to increase hatching rates in mouse embryos (Menino and O'Claray 1986).

We were unable to assess the effects of the combination of IGF-II, uPA and plasminogen on fetal and placental weights at term and on postnatal outcomes. This

may be due to the strain of mouse chosen for the study. C57Bl6 were used as both embryo donors and recipients. The C57Bl6 strain has a well-characterised obese phenotype, particularly when exposed to high fat diets, and is prone to developing diabetes and insulin resistance (Surwit, Kuhn et al. 1988; Lin, Thomas et al. 2000), resulting from a naturally occurring deletion in the Nnt (nicotinamide nucleotide transhydrogenase) gene (Toye, Lippiat et al. 2005; Freeman, Shimomura et al. 2006; Freeman, Hugill et al. 2006). In addition, pregnancy perturbations in C57Bl6 mice have been shown to increase postnatal obesity (White, Johansson et al. 2004). This strain was therefore chosen to enable investigation of the effects of embryo culture conditions on both pregnancy outcomes and on postnatal phenotype. However, embryos from C57Bl6 mothers appear to be very sensitive to the adverse effects of superovulation and *in vitro* culture. They have decreased blastocyst development *in vitro* compared to other strains, and following embryo transfer there is a high rate of fetal loss during pregnancy. Therefore future work using a different strain of mice will be performed to determine the effects of the combination treatment on late gestation pregnancy outcomes and postnatal health.

Nonetheless, it seems that the combination of all three factors, IGF-II, uPA and plasminogen helps to overcome some of the adverse effects of *in vitro* culture. We hypothesise that these three molecules form a complex with the IGF2R and the uPAR to promote blastocyst hatching and subsequent implantation and placentation. Indeed, previous work has shown that the IGF2R is functional during early embryo development since IGF-II promotes cleavage rates and glucose uptake by the blastocyst, only when IGF2R is present (Pantaleon, Jericho et al. 2003). However, further work is required to determine whether the formation of this complex with

IGF2R and uPAR along with their ligands, is implicated in blastocyst hatching and implantation.

In conclusion, this new media formulation which includes IGF-II, uPA and plasminogen in combination increases the proportion of blastocysts hatching and increases implantation and pregnancy rates. Although future work is required to determine its effects on fetal and placental weights in late gestation, pregnancy outcome and maternal and postnatal health, we envisage that this new media formulation may one day be used for embryo culture in ART.

Chapter 3
New Light on Early Post-Implantation Pregnancy in the Mouse: Roles for Insulin-Like Growth Factor-II?

3.1 Introduction

The human placenta is highly invasive (Lala and Graham 1990; Foidart, Hustin et al. 1992). Trophoblast cells breach the uterine luminal epithelium and colonise the endometrium and its vasculature to sequester a blood supply for the developing placenta. Impaired trophoblast invasion has been implicated in several complications of pregnancy such as unexplained miscarriage (Khong, Liddell et al. 1987), preeclampsia and intrauterine growth restriction (Khong, De Wolf et al. 1986), placental abruption (Dommisse and Tiltman 1992), pre-term labour with intact membranes (Kim, Bujold et al. 2003), premature rupture of the membranes (Kim, Chaiworapongsa et al. 2002) and stillbirth (Smith, Crossley et al. 2004). Insufficient trophoblast invasion and vascular remodelling are thought to reduce maternal blood flow to the placenta (Jauniaux, Hempstock et al. 2003).

Many growth factors have been implicated in placentation. Insulin-like growth factor (IGF)-II is a key mediator of trophoblast invasion in humans (Irving and Lala 1995). IGF-II is a polypeptide of approximately 7.5 kDa that has structural homology with pro-insulin, as well as with IGF-I (Rinderknecht and Humbel 1978; O'Dell and Day 1998). In addition to its mitogenic actions, IGF-II promotes differentiation and migration and inhibits apoptosis, all of which are essential for placental development (Han and Carter 2000).

IGF-II is able to bind to three cell surface receptors, the type I IGF receptor (IGF1R), the type II IGF receptor (IGF2R) and the insulin receptor, but it binds to the IGF2R with the highest affinity (Rechler and Nissley 1985). Current evidence suggests that most of the effects of IGF-II on growth and differentiation are elicited by activation of the IGF1R, a tyrosine kinase transmembrane receptor (Jones and Clemmons 1995).

The IGF2R is identical to the cation independent mannose-6-phosphate receptor (CI-M6PR) (Morgan, Edman et al. 1987; Kiess, Blickenstaff et al. 1988). Binding of IGF-II to the IGF2R is thought to result primarily in clearance of the IGFs from plasma and tissue fluids (Han and Carter 2000). However, there is evidence to suggest that IGF-II, upon binding to the IGF2R, can stimulate an intracellular signalling pathway using a Gi-coupled receptor (Murayama, Okamoto et al. 1990; Ikezu, Okamoto et al. 1995) and mitogen activated protein kinase (MAPK) (McKinnon, Chakraborty et al. 2001).

Gene ablation studies in mice suggest that IGF-II and its receptors are important regulators of placental growth and differentiation. Heterozygous mice carrying an *Igf2* gene disrupted by targeting were born approximately 60% of the size of their wild-type littermates (DeChiara, Efstratiadis et al. 1990), and had decreased placental weight (Lopez, Dikkes et al. 1996). Similarly, deletion of an *Igf2* transcript (P0) specifically expressed in the trophoblast cells of the placental labyrinthine layer, reduced placental growth and transport followed by reduced fetal growth late in gestation (Constancia, Hemberger et al. 2002; Sibley, Coan et al. 2004). Conversely, mice inheriting a non-functional *Igf2r* gene from their mothers die at or near the time of birth, as a consequence of major cardiac abnormalities (Lau, Stewart et al. 1994) but are completely rescued when they carry a second mutation eliminating either *Igf2* or *Igf1r* (Ludwig, Eggenschwiler et al. 1996). The *Igf2r* null mice are approximately 30-40% larger than their normal siblings at birth, have increased placental weight and increased serum concentrations and tissue levels of IGF-II and several IGF binding proteins (IGFBPs) (Lau, Stewart et al. 1994; Ludwig, Eggenschwiler et al. 1996). This genetic evidence suggests that regulation of IGF-II concentrations by IGF2R is essential to prevent fetal overgrowth. By contrast, *Igf1r* null mice have reduced fetal weight in late gestation with no change in placental weight, suggesting that the

function of IGF-II in the placenta is mediated by a receptor other than IGF1R (Baker, Liu et al. 1993). Louvi et al. demonstrated that IGF-II effects on fetal growth are mediated by the insulin receptor but provided no information on those in the placenta (Louvi, Accili et al. 1997).

In humans, IGF-II is differentially expressed in the two layers of the villous trophoblast in the placenta. The inner layer of cytotrophoblast cells (CTB) expresses IGF-II, while the outer syncytiotrophoblast (STB) does not (Redline, Chernicky et al. 1993; Han, Bassett et al. 1996). Copious *Igf2* mRNA was also identified in the columns of CTBs and extravillous cytotrophoblast cells (EVT) invading the decidua and its vasculature, (Hamilton, Lysiak et al. 1998); (Han, Bassett et al. 1996). Comparatively, IGF-II is expressed in the murine placenta in both the junctional zone and the labyrinth on days 9.5-12.5 (Redline, Chernicky et al. 1993; Han and Carter 2000; Tycko and Efstratiadis 2002). After this, expression of IGF-II in spongiotrophoblast cells of the junctional zone decreases as the new invasive trophoblast glycogen cell population emerges and strongly expresses IGF-II (Redline, Chernicky et al. 1993).

Despite the compelling evidence that IGF-II plays an important role in placental growth, structural development and transport in late gestation in mice, there have been few studies examining its role early in the post-implantation period when trophoblast cells are at their most invasive, when essential decidual remodelling and angiogenesis are maximal and when placental morphogenesis is initiated. In humans, the events during this phase of placental development are critical to subsequent pregnancy outcome (Jauniaux, Hempstock et al. 2003). The aims of the current study therefore, were to localise IGF-II and the IGF receptors, IGF1R and

IGF2R, in early murine implantation sites, with particular focus on invading trophoblasts and the decidual vasculature.

3.2 Materials and Methods

3.2.1 Mice

This study was approved by the Animal Ethics Committee of the University of Adelaide. C57BL/6 males and CBA/F1 females were obtained from the University of Adelaide Central Animal House. All mice were housed in clean conventional conditions on a cycle of 12 hours light, 12 hours dark and received food and water ad libitum. Two females were caged with each male overnight and checked for the presence of vaginal copulatory plugs the following morning. The morning that the vaginal plug was found was designated as day 0.5 of pregnancy.

3.2.2 Immunohistochemistry

Histological analyses of implantation sites at each day of days 5.5 to 10.5 of development obtained from three different mice per day were performed. Mice were killed by cervical dislocation. Uterine horns were removed and fixed in 4% (g/v) paraformaldehyde, 2.5% (g/v) polyvinyl pyrrolidone in 70 mmol/l sodium phosphate buffer overnight, before being divided into individual implantation sites. These were dehydrated in alcohol, embedded in paraffin blocks and cut into 6μm serial sagittal sections.

Every tenth section was stained with hematoxylin and eosin (H&E) for general morphology and to identify slides in which transverse sections through the implantation sites were present. These sections were processed by immunohistochemistry to localise cytokeratin in trophoblast cells (Zymed, San

Francisco, USA, pAb, rabbit anti-human cytokeratin, 1:25), endothelial cells (MTS-12, kind gift of Professor Richard Boyd, Department of Pathology and Immunology, Monash University, Melbourne, Australia, mAb, rat anti-mouse MTS-12 supernatant, undiluted), smooth muscle actin (Sigma, St. Louis, USA, mAb, anti-mouse smooth muscle actin, 1:800), IGF-II (R&D Systems, Minneapolis, USA, pAb, goat anti-human IGF-II, 1:20), IGF1R (Santa Cruz Biotechnology, California, USA, pAb, rabbit-anti human IGF1Rα, 1:10), and IGF2R (kind gift of Dr Carolyn Scott, Kolling Institute of Medical Research, Sydney, Australia, pAb, rabbit anti-rat IGF2R, 1:100). After deparaffinization and hydration, the sections were subjected to antigen retrieval by 0.01% pronase (Sigma, St Lois, USA) treatment for 10 minutes at 37°C (cytokeratin, MTS-12, IGF-II and IGF1R labelling only). After blocking for endogenous peroxidase activity (3% hydrogen peroxide for 30 minutes) tissues were washed in phosphate buffered saline (PBS) and treated with 1% bovine serum albumin (BSA; Sigma, St Louis, USA) and 10% serum in PBS for 10 minutes to block non-specific binding, before being incubated overnight with the primary antibody.

After three washes in PBS, the sections were incubated for 1 hour with their relevant biotinylated secondary antibody. For MTS-12 rabbit anti-rat IgG, for cytokeratin and IGF2R, F (ab')2 fragments of swine anti-rabbit IgG, for smooth muscle actin, rabbit anti-mouse IgG, for IGF-II, rabbit anti-goat IgG and for IGF1R goat anti-rabbit IgG secondary antibodies were employed (Dako Corporation, Carpentaria, CA, USA). Sections were washed again and incubated for 1 hour with horseradish peroxidase (HRP) conjugated with streptavidin (Rockland, Gilbertsville, PA, 1:500). The site of antibody binding was visualised with diaminobenzidine (DAB; Sigma, St Louis, USA) and sections were counterstained with hematoxylin. The primary and secondary antibodies were diluted in a solution containing 1% BSA, 10% serum and PBS.

Negative controls used either antibody diluent or irrelevant IgG in place of the primary antibodies.

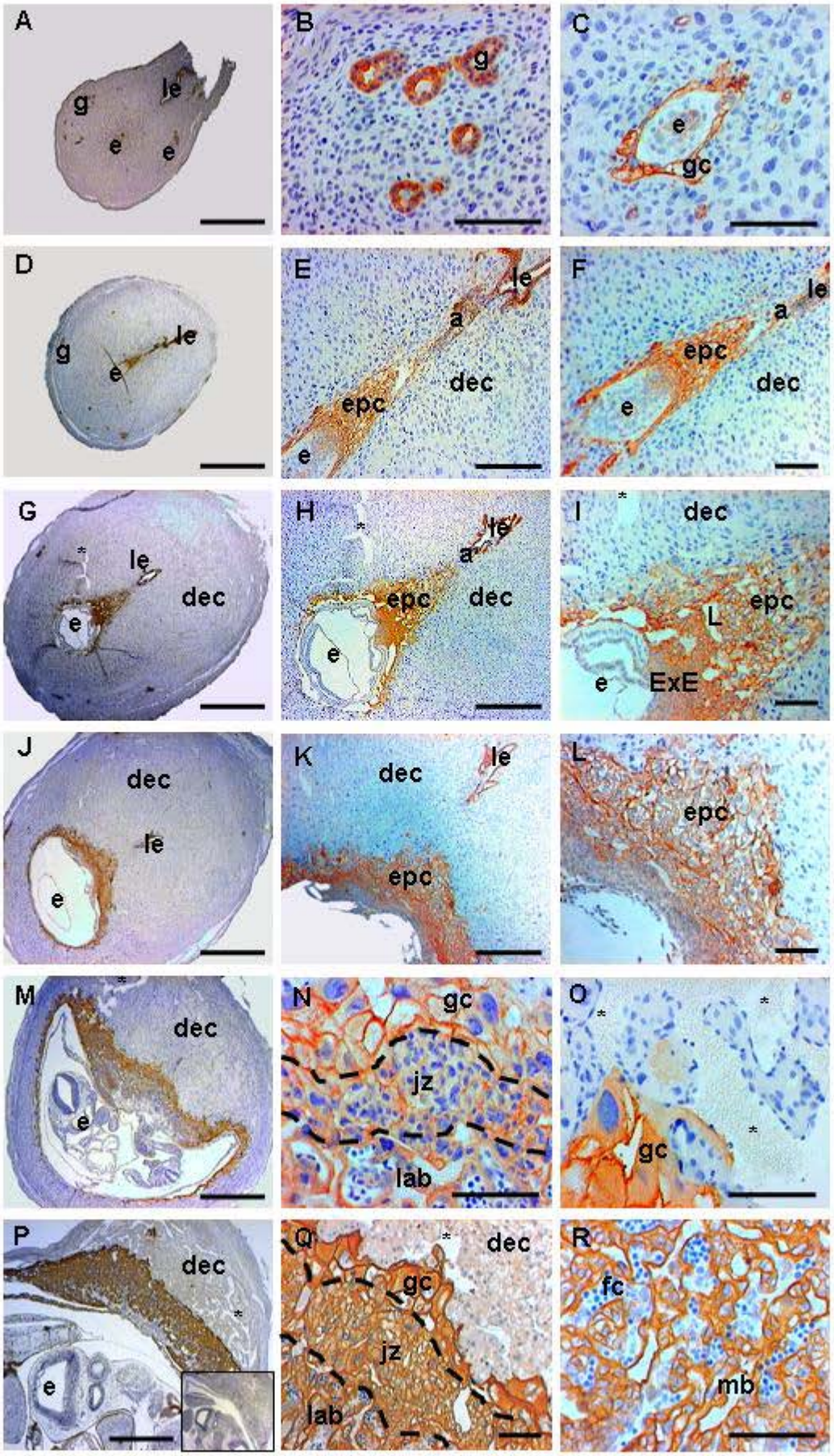
3.3 Results

3.3.1 Early murine placental morphogenesis

It was first necessary to localise trophoblast cells in the sections and thus an atlas of early mouse placentation has been provided (Figure 3-1). Antibodies raised against cytokeratin, immunostained all trophoblast cells as well as the uterine luminal and glandular epithelia (Figure 3-1A, B).

On day 4.5, the blastocyst attaches to the uterine epithelium in a uterine crypt and by day 5.5, the peripheral, flattened trophectoderm cells of the embryo differentiate into trophoblast giant cells (TGCs), appearing as a single layer of cells surrounding the embryo (Figure 3-1 A, C). On day 6.5 (Figure 3-1 D-F), the polar trophectoderm proliferates to form the extraembryonic ectoderm (ExE), which apposes the epiblast of the embryo, and distal to the ExE the ectoplacental cone (EPC) is observed. Trophoblast cells migrate irregularly from the EPC and intercellular lacunae are formed, within which maternal blood has been found (Muntener and Hsu 1977) (Figure 3-1 I). By day 7.5, the EPC is clearly visible in the sections (Figure 3-1 G-I); the trophoblast cells in the outer regions of the EPC differentiate into secondary TGCs and phagocytose the presumably apoptotic luminal epithelium at the mesometrial pole. The luminal epithelium has disappeared by day 9.5 of pregnancy (Figure 3-1 J, M). TGCs form the interface between the placenta and the maternal decidua throughout early to mid pregnancy. By day 9.5 the three different regions of the mouse chorioallantoic placenta begin to form (Figure 3-1 M-O), and are further developed by day 10.5 (Figure 3-1 P-R). Adjacent to the chorionic plate is the

Figure 3-1 Cytokeratin immunostaining in day 5.5-10.5 murine implantation sites
(**A**) Day 5.5 of gestation. (**B**) Day 5.5 uterine glands, displaying cytokeratin immunostaining of the glandular epithelium. (**C**) Day 5.5 conceptus, with a layer of TGC surrounding the murine embryo. (**D**) Day 6.5 of gestation. (**E**, **F**) Day 6.5 conceptus. The trophoblast opposite to the ICM have proliferated giving rise trophoblast stem cells to make up the EPC. At this stage the apoptotic uterine luminal epithelium can be observed and is thought to be phagocytosed by the trophoblast giant cells. (**G**) Day 7.5 of gestation. (**H**) Day 7.5 implantation site again displaying the embryo, EPC and luminal epithelium. Note the dilation of the maternal vessels at this stage. (**I**) Day 7.5 of gestation, a higher magnification view of (**H**) showing the EPC with the intercellular lucanae which contain maternal blood. (**J**) Day 8.5 of gestation. (**K**) Day 8.5 EPC with adjacent mesometrial decidua and uterine lumen. (**L**) A higher magnification view of the day 8.5 EPC. (**M**) Day 9.5 of gestation. (**N**) Day 9.5 placenta. The three layers of the placenta can now be seen. At the placental/decidual border is the TGC layer, which overlies the junctional zone, and the labyrinth. (**O**) Day 9.5 TGCs lying within dilated maternal vessels, which are still lined by endothelium. (**P**) Day 10.5 of gestation and the negative control (inset). (**Q**) The placenta on day 10.5 of gestation, again showing the three layers of the placenta and several TGCs lying within maternal blood vessels. (**R**) A higher magnification view of the labyrinth, which consists of maternal blood spaces lined by cytokeratin immunostained trophoblast cells, and fetal capillaries. a, apoptotic uterine luminal epithelium; dec, decidua; e, embryo; epc, ectoplacental cone; ExE extra embryonic ectoderm; fc, fetal capillary; g, uterine glands; gc, giant cell layer; jz, junctional zone; L, lucanae; lab, labyrinthine zone; le, uterine luminal epithelium; mb, maternal blood space; *, dilated maternal blood vessel.


A
g
le
e
e
B
g
C
e
gc
D
g
le
e
E
le
a
epc
dec
e
F
le
a
epc
dec
e
G
*
le
dec
e
H
*
le
a
epc
dec
e
I
*
dec
epc
L
e
ExE
J
dec
le
e
K
dec
le
epc
L
epc
M
*
dec
e
N
gc
jz
lab
O
*
*
*
gc
P
dec
*
e
Q
*
dec
gc
jz
lab
R
fc
mb

labyrinthine layer (Figure 3-1 R), consisting of cytokeratin positive trophoblast cells, fetal capillaries and maternal blood spaces. Distal to the labyrinth is the junctional zone, which is comprised of maternal blood spaces and spongiotrophoblast cells but contains no fetal blood vessels. Between the maternal decidua and the junctional zone is the zone of giant cells.

3.3.2 Trophoblast invasion

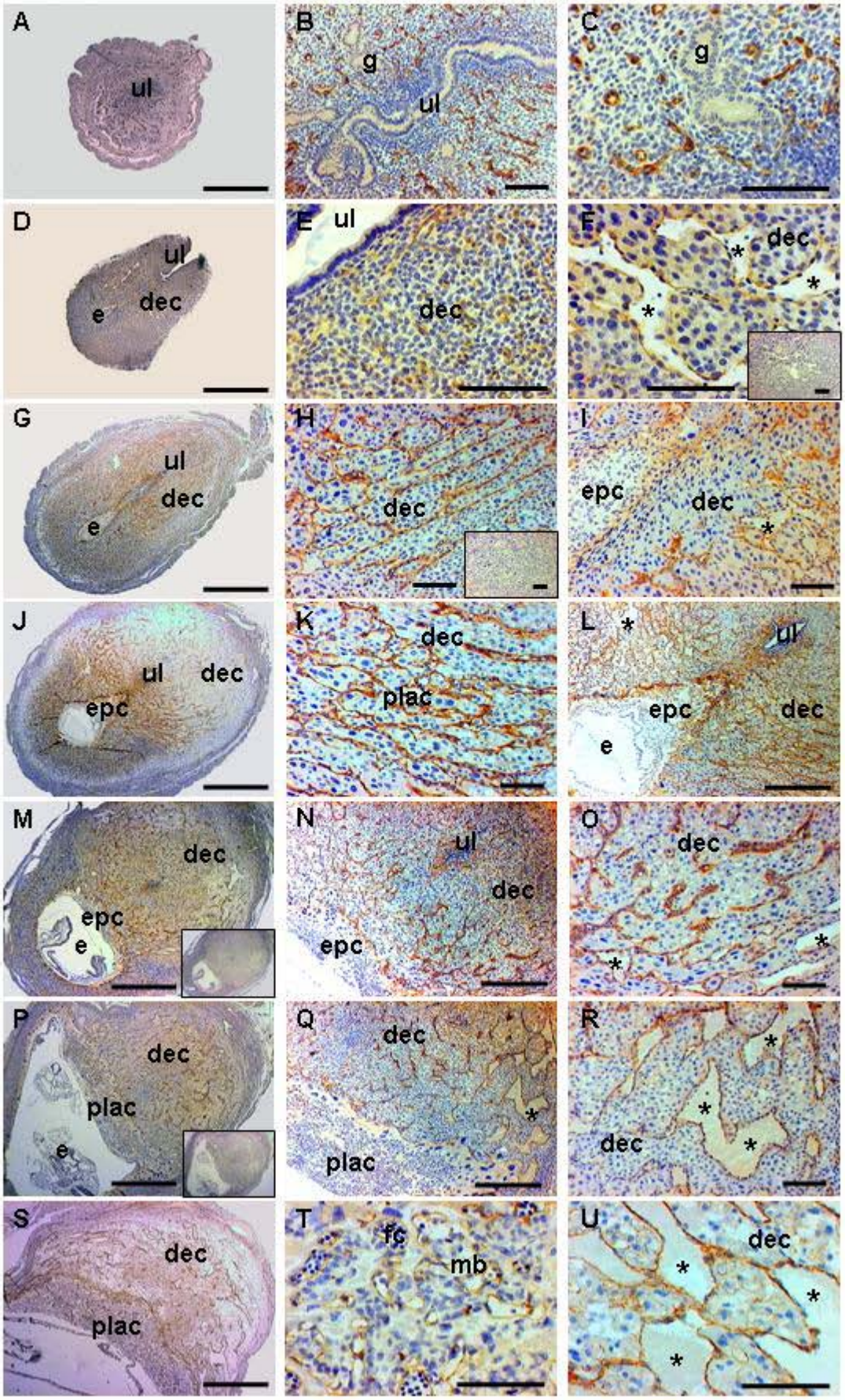
Throughout early murine pregnancy there is no evidence of trophoblast cells invading individually into the maternal tissue. Instead, the TGCs, which are at the interface between the placenta and the maternal tissue, remain in close contact with each other throughout early post-implantation development. There is some evidence of TGCs lying within maternal blood vessels (Figure 3-1 O), but these are always observed adjacent to the TGC layer. All maternal blood spaces beneath this layer are lined by trophoblast cells and not endothelium (Figure 3-1 R and Figure 3-2 T).

3.3.3 Decidual vascularisation

The changes occurring in the decidual vasculature during these early stages of pregnancy are extraordinary and were visualised using an MTS-12 antibody to localise endothelium (Figure 3-2). On day 5.5, the uterine tissue in the inter-implantation sites (Figure 3-2 A-C) and in the decidua (Figure 3-2 D-F) is traversed by an intricate network of capillaries. By day 7.5 these capillaries have become dilated and are much more organised, seen as cords of endothelial lined tubes tracking through the mesometrial decidua, through the glycogen rich region, to supply the conceptus (Figure 3-2 G-I). There are only comparatively very few capillaries found in the anti-mesometrial decidua and they do not undergo this extensive transformation. By day 10.5, the mesometrial decidual capillaries are

Figure 3-2 Localisation of endothelial cells using MTS-12 antibody in early murine implantation sites

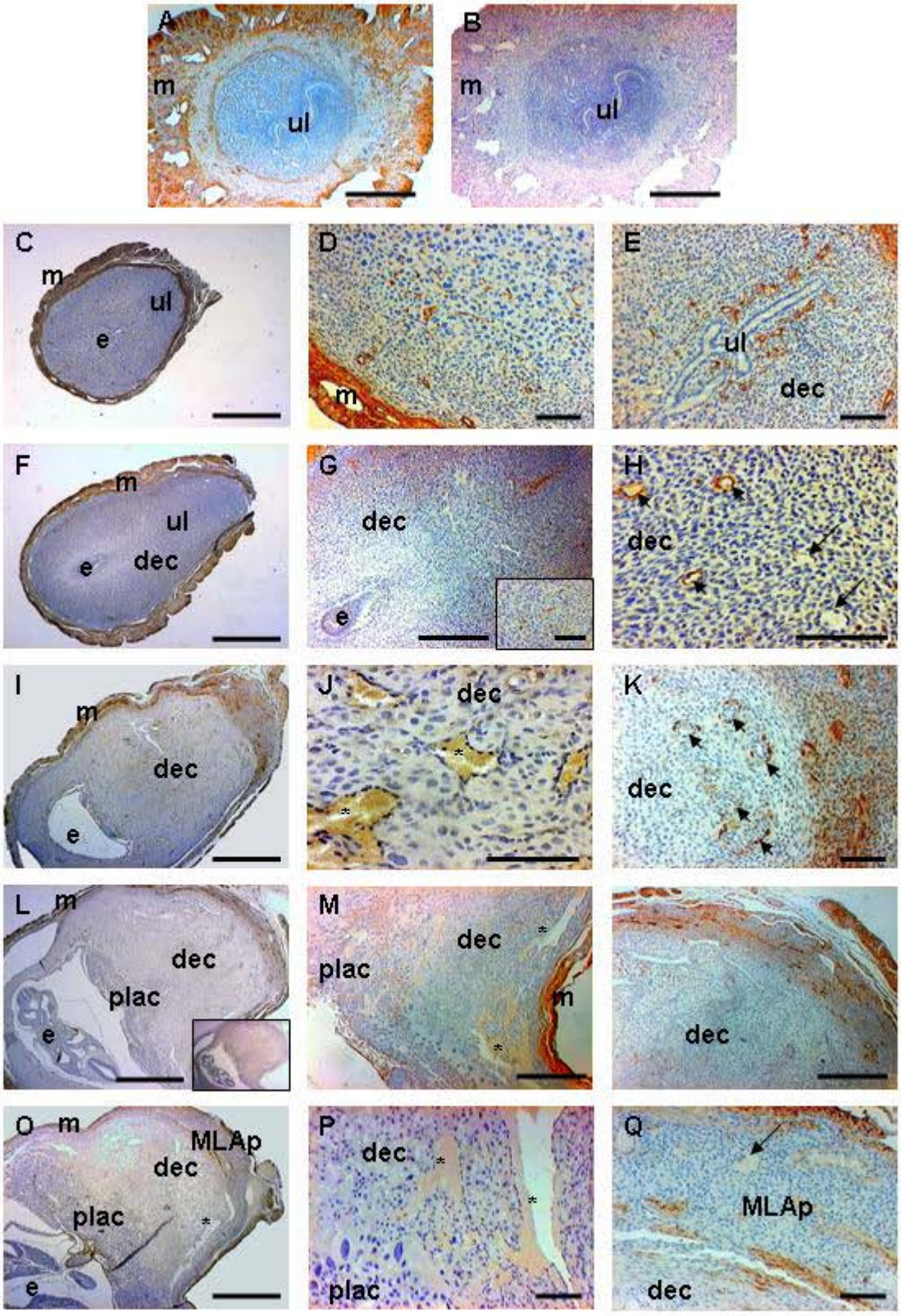
(**A**) Day 5.5 of gestation, inter-implantation site. (**B**) Day 5.5 inter-implantation site non-decidualised stroma and uterine lumen. (**C**) Higher powered magnification of **B**. (**D**) Day 5.5 implantation site. (**E**) Day 5.5 of gestation. MTS-12 immunostained maternal vessels surrounding the uterine lumen. (**F**) MTS-12 immunostained dilated maternal vessels in the mesometrial decidua and the negative control (Inset). (**G**) Day 6.5 of gestation. (**H**) Day 6.5 implantation site with cords of endothelial lined vessels traversing the mesometrial decidua and the negative control (inset). (**I**) Day 6.5 maternal vessels adjacent to the conceptus. (**J**) Day 7.5 of gestation. (**K**) maternal vessels of the day 7.5 mesometrial decidua. (**L**) Day 7.5 maternal vessels adjacent to the conceptus. (**M**) Day 8.5 of gestation and the negative control (inset). (**N**) Day 8.5 placenta and the adjacent vessels of the mesometrial decidua. (**O**) Higher magnification view of the day 8.5 mesometrial vessels. (**P**) Day 9.5 of gestation and the negative control (inset). (**Q**) Day 9.5 placenta and the adjacent vessels of the mesometrial decidua. (**R**) Dilated maternal vessels of the day 9.5 mesometrial decidua. (**S**) Day 10.5 of gestation. (**T**) Day 10.5 placental labyrinth with MTS-12 immunostaining in the endothelium of the fetal capillaries. (**U**) Day 10.5 dilated maternal vessels in the mesometrial decidua. dec, decidua; e, embryo; epc, ectoplacental cone; fc, fetal capillaries; g, uterine glands; mb, maternal blood space; plac, placenta; ul, uterine luminal epithelium; *, dilated maternal blood vessel.


A
ul
B
g
ul
C
g
D
ul
dec
e
E
ul
dec
F
dec
*
*
*
G
ul
dec
e
H
dec
I
epc
dec
*
J
ul
dec
epc
K
dec
plac
L
*
ul
epc
dec
e
M
dec
epc
e
N
ul
dec
epc
O
dec
*
*
P
dec
plac
e
Q
dec
*
plac
R
*
*
dec
*
S
dec
plac
T
fc
mb
U
dec
*
*
*

extremely dilated, presumably permitting increased blood flow to the placenta (Figure 3-2 U). Throughout these early stages of pregnancy, there is no evidence of individual TGCs migrating into the maternal tissue, suggesting that the remodelling of the vasculature in the decidua occurs independently of trophoblast cells. On days 8.5-10.5, TGCs at the interface between the decidua and the placenta are present within the capillaries (Figure 3-2 O). Maternal blood spaces beneath the TGC layer are lined by trophoblast cells (Figure 3-2 R) and the only endothelium in the labyrinth is that surrounding the fetal capillaries which are easily identified by the presence of nucleated red blood cells within the lumina (Figure 3-2 T).

To identify the vessels in the decidua that are lined by smooth muscle we used an antibody raised against smooth muscle actin (Figure 3-3). Throughout early pregnancy the myometrium that surrounds the decidua is immunoreactive for smooth muscle actin. In the day 5.5 inter-implantation sites there is little to no smooth muscle immunostaining in the uterine stroma (Figure 3-3 A-B) where strong MTS-12 immunoreactivity was observed (Figure 3-3 A-C). However, in the implantation site there is smooth muscle immunostaining around some of the vessels in the outer anti-mesometrial undifferentiated stroma but none closest to the embryo in the primary decidual zone (PDZ) (Figure 3-3 C-D). There is also immunostaining in vessels around the uterine lumen in the mesometrial decidua (Figure 3-3 E). Soon after this time the smooth muscle in the anti-mesometrial decidual vessels is lost and only immunoreactivity of vessels in the mesometrial decidua remains. By day 7.5, the remaining mesometrial luminal epithelium appears to undergo apoptosis and the surrounding region appears to be remodelled, so by day 8.5, there are only a few remaining maternal spiral arterioles that contain smooth muscle. The dilated maternal vessels seen in the mesometrial decidua at this time are lined only by

Figure 3-3 Smooth muscle actin antibody immunostaining in day 5.5 to 10.5 implantation sites
(**A**) Day 5.5 inter-implantation site. (**B**) Day 6.5 inter-implantation site negative control. (**C**) Day 5.5 implantation site. (**D**) Higher magnification view of day 5.5 antimesometrial decidua. (**E**) Higher magnification view of day 5.5 uterine lumen and mesometrial decidua. (**F**) Day 6.5 of gestation. (**G**) Higher power view of Day 6.5 conceptus and adjacent mesometrial decidua. Inset: Higher magnification view of the smooth muscle immunostaining in the maternal vessels in the outer mesometrial decidua. (**H**) Higher power view of day 6.5 vessels in the outer mesometrial decidua. The spiral arteries show smooth muscle actin immunoreactivity while other vessels do not. (**I**) Day 8.5 of gestation. (**J**) Higher magnification view of the dilated maternal vessels of the mesometria decidua. These vessels display no smooth muscle actin immunoreactivity. (**K**) Spiral arterioles of the outer mesometrial decidua on day 8.5. (**L**) Day 9.5 of gestation. Inset: negative control. (**M**) Higher magnification view of the placenta and mesometrial decidua on day 9.5. (**N**) Higher magnification view of the outer antimesometrial decidua. (**O**) Day 10.5 of gestation. (**P**) Higher magnification view of the day 10.5 dilated maternal vessels in the mesometrial decidua, which display a lack of smooth muscle actin immunoreactivity. (**Q**) Higher magnification view of the day 10.5 MLAp. dec, decidua; e, embryo; m, myometrium; MLAp, mesometrial lymphoid aggregate of pregnancy; plac, placenta; ul, uterine luminal epithelium; arrow, maternal vessels with no smooth muscle immunoreactivity; arrowhead, spiral arteriole displaying smooth muscle immunoreactivity; *, dilated maternal blood vessel (with no smooth muscle layer).

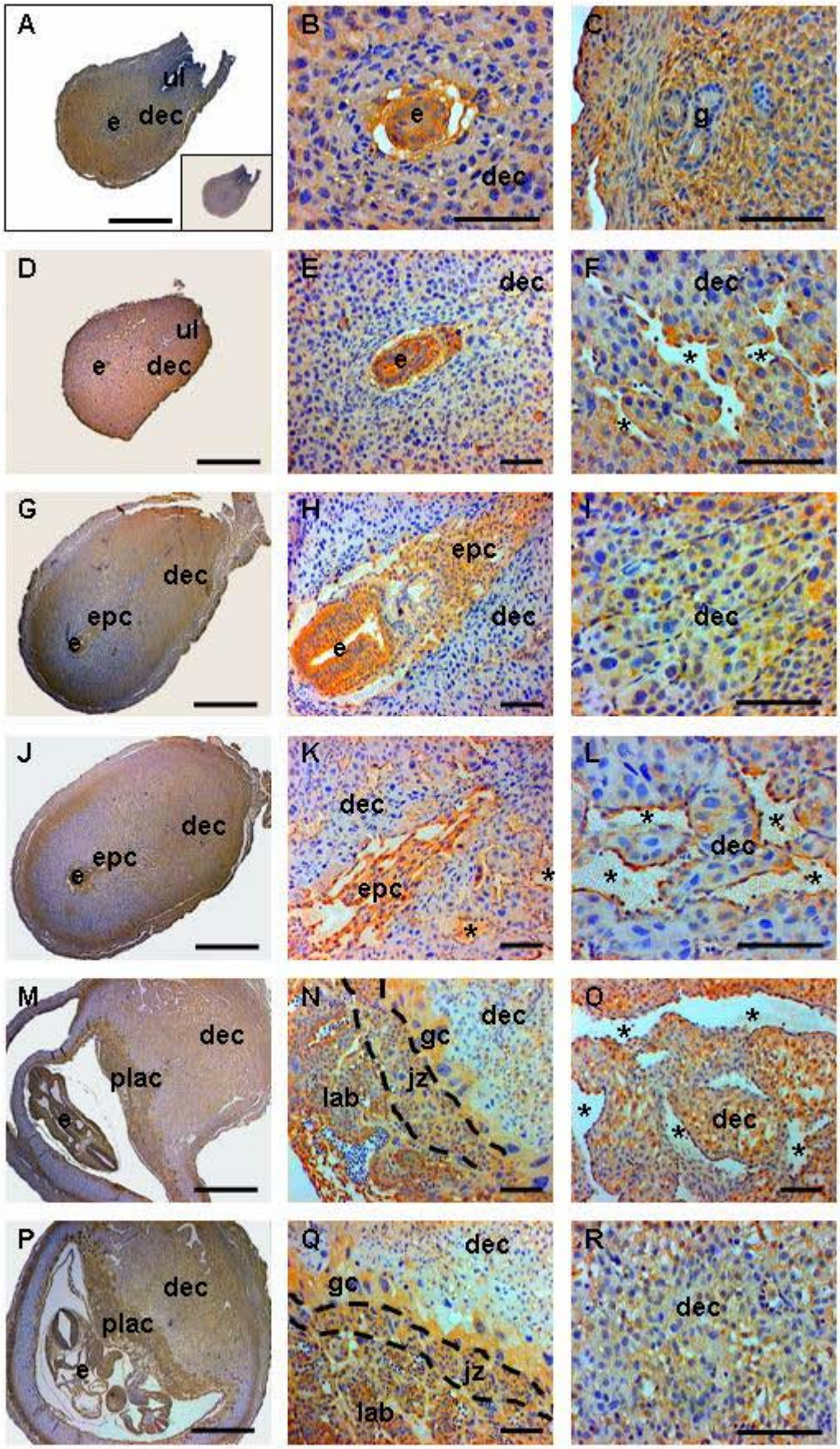

A
m
ul
B
m
ul
C
m
ul
e
D
m
E
ul
dec
F
m
ul
e
dec
G
dec
e
H
dec
I
m
dec
e
J
dec
*
*
K
dec
L
m
dec
plac
e
M
dec
*
plac
m
*
dec
O
m
MLAp
dec
plac
*
e
P
dec
*
*
plac
Q
MLAp
dec

endothelium (Figure 3-3 IK). As expected, on day 9.5 and 10.5 there is also smooth muscle immunostaining in the mesometrial lymphoid aggregate of pregnancy (MLAp), formerly known as the metrial gland (Figure 3-3 N, Q).

3.3.4 IGF-II protein expression

IGF-II is expressed by the conceptus throughout early pregnancy (Figure 3-4). From days 5.5 to 8.5, IGF-II is localised to the cytoplasm of all trophoblast cells, including the TGCs and those in the ExE and the EPC (Figure 3-4). On days 9.5-10.5 the trophoblasts of the labyrinthine region, as well as the junctional zone, also label strongly for IGF-II (Figure 3-4 N, Q). The entire murine embryo displays very strong cytoplasmic immunostaining for IGF-II throughout early pregnancy. Although the decidua immediately surrounding the conceptus does not label for IGF-II, there is cytoplasmic IGF-II immunostaining in the peripheral decidua in the mesometrial, but not in the anti-mesometrial, decidua from day 5.5 onwards (Figure 3-4). Interestingly, there is IGF-II immunostaining in the decidual endothelium close to the developing placenta. This endothelial immunoreactivity is only associated with those vessels that appear to undergo remodelling on days 6.5 to 8.5 of pregnancy.

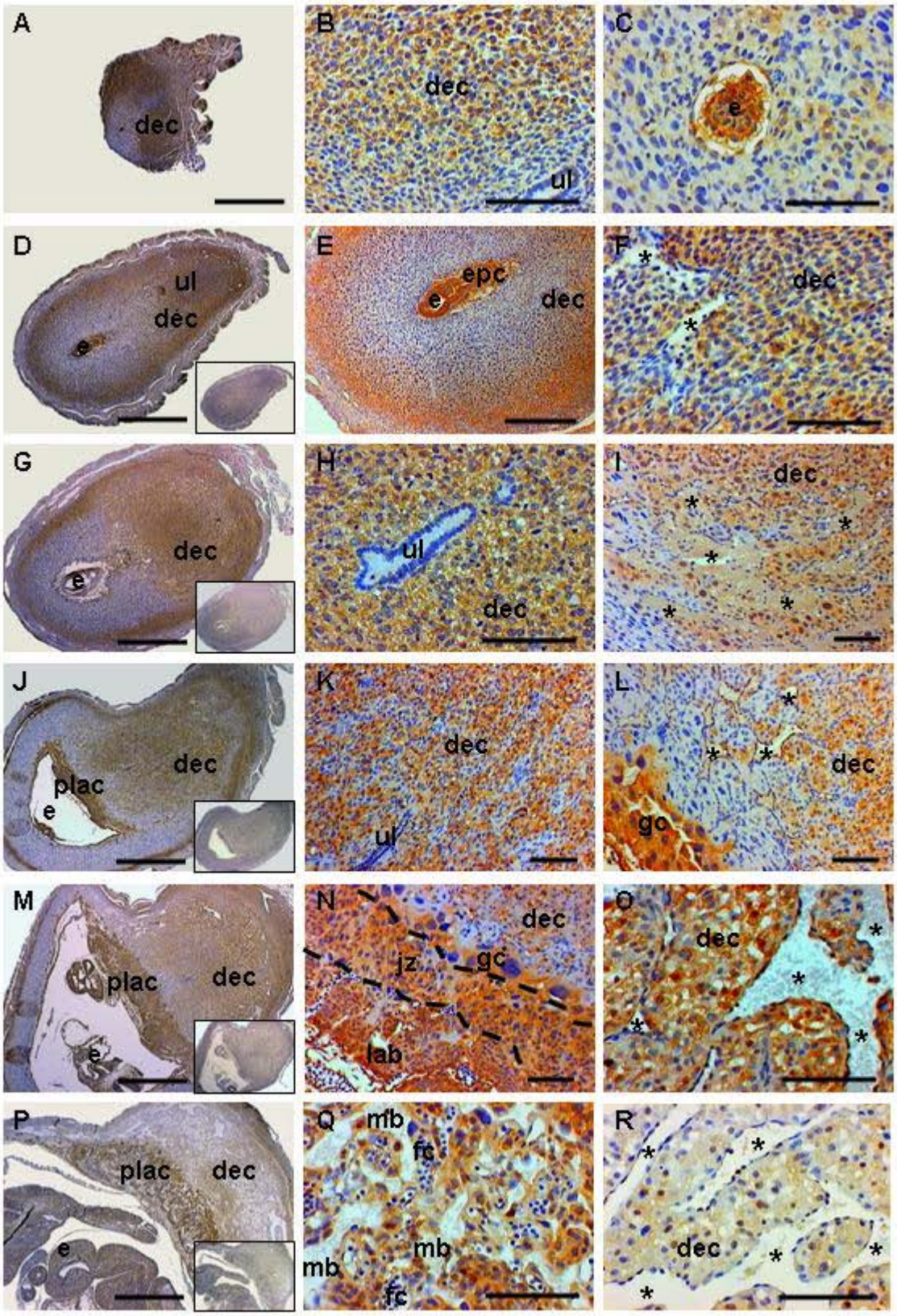
Figure 3-4 IGF-II immunostaining in day 5.5 – 10.5 murine implantation sites
(**A**) Day 5.5 of gestation. Inset: day 5.5 negative control. Scale bar = 800 μm. (**B**) Higher magnification view of day 5.5 conceptus and surrounding decidua. Scale bar = 100 μm. (**C**) Higher magnification view of day 5.5 antimesometrial region. Scale bar = 100 μm. (**D**) Day 6.5 of gestation. Scale bar = 800 μm. (**E**) Higher magnification view of day 6.5 conceptus and surrounding decidua. Scale bar = 100 μm. (**F**) Higher magnification view of dilated maternal vessels in the mesometrial decidua. Scale bar = 100 μm. (**G**) Day 7.5 of gestation. Scale bar = 800 μm. (**H**) Day 7.5 embryo and EPC. Scale bar = 100 μm. (**I**) Cords of endothelial cells in the mesometrial decidua on day 7.5. Scale bar = 100 μm. (**J**) Day 8.5 of gestation. Scale bar = 800 μm. (**K**) Higher magnification view of the day 8.5 EPC and surrounding decidua. Scale bar = 100 μm. (**L**) Dilated maternal vessels in the day 8.5 mesometrial decidua. Note the IGF-II immunostaining in the endothelium at this stage. Scale bar = 100 μm. (**M**) Day 9.5 of gestation. Scale bar = 800 μm. (**N**) Day 9.5 placenta. Scale bar = 100 μm. (**O**) Dilated maternal vessels in the day 9.5 mesometrial decidua. Scale bar = 100 μm. (**P**) Day 10.5 of gestation. Scale bar = 800 μm. (**Q**) Day 10.5 placenta. Scale bar = 100 μm. (**R**) Day 10.5 mesometrial decidua. Scale bar = 100 μm. dec, decidua; e, embryo; epc, ectoplacental cone; g, uterine gland; gc, giant cell layer; jz, junctional zone; lab, labyrinthine zone; plac, placenta; ul, uterine lumen; *, dilated maternal blood vessel.


A
ul
e
dec
B
e
dec
C
g
D
ul
e
dec
E
dec
e
F
dec
*
*
*
G
dec
epc
e
H
epc
dec
e
I
dec
J
dec
epc
e
K
dec
epc
*
*
L
*
*
dec
*
*
M
dec
plac
e
N
l
dec
gc
jz
lab
Q
*
*
*
dec
*
*
P
dec
plac
e
Q
dec
gc
jz
lab
R
dec

3.3.5 IGF1R protein expression

Strong cytoplasmic IGF1R immunostaining is present in the embryo throughout early murine pregnancy (Figure 3-5). All trophoblast cells of the early murine placenta also stain strongly for IGF1R, however TGCs on the anti-mesometrial pole show little to no staining. There is strong cytoplasmic IGF1R immunoreactivity in the outer anti-mesometrial decidua and in the mesometrial decidua on days 5.5 and 6.5 of pregnancy (Figure 3-5 A-F). No immunostaining is observed in the decidua immediately surrounding the conceptus. This pattern of staining continued on day 7.5 with the addition of nuclear IGF1R immunoreactivity in the mesometrial decidua in the glycogen rich region surrounding the dilated maternal vessels (Figure 3-5 I). On days 8.5 and 9.5 some endothelial IGF1R immunostaining is also observed in addition to that observed on the previous days (Figure 3-5 L, O). On Day 10.5, the pattern of IGF1R immunostaining in the mesometrial decidua declines and what staining is visible, is confined to the central decidua closest to the TGCs and to the spiral arterioles in the outer anti-mesometrial tissue (Figure 3-5 P-R). This immunostaining is predominantly cytoplasmic. There is also no longer any IGF1R staining in the endothelium and little to no immunostaining in the decidua surrounding these dilated maternal vessels (Figure 3-5 R).

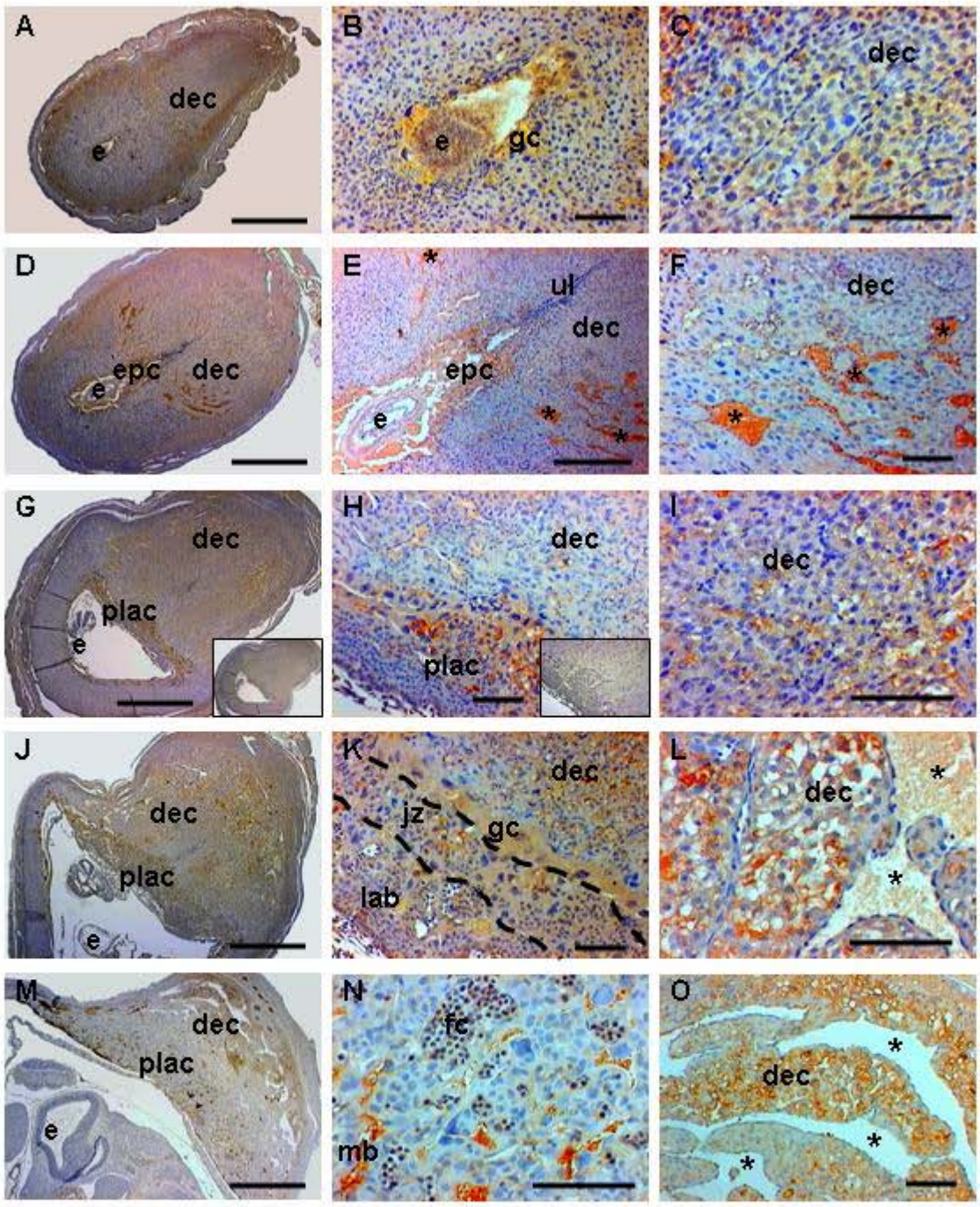
Figure 3-5 IGF1R immunostaining in day 6.5 – 10.5 murine implantation sites
(**A**) Day 5.5 of gestation. Scale bar = 800 μm. (**B**) Day 5.5 mesometrial decidua surrounding the uterine lumen. Scale bar = 100 μm. (**C**) Day 5.5 conceptus. Scale bar = 100 μm. (**D**) Day 6.5 of gestation. Inset: negative control. Scale bar = 800 μm. (**E**) Day 6.5 conceptus and antimesometrial region. Scale bar = 400 μm. (**F**) Day 6.5 dilated maternal vessels in the mesometrial decidua. Scale bar = 100 μm. (**G**) Day 7.5 implantation site. Inset: negative control. Scale bar = 800 μm. (**H**) Day 7.5 mesometrial decidua. Scale bar = 100 μm. (**I**) Higher magnification view of the dilated maternal vessels and the surrounding mesometrial decidua on day 7.5. Scale bar = 100 μm. (**J**) Day 8.5 of gestation. Inset: negative control. Scale bar = 800 μm. (K) Day 8.5 mesometrial decidua and uterine lumen. Scale bar = 100 μm. (**L**) dilated maternal vessels in the mesometrial decidua and the adjacent TGC layer. Scale bar = 100 μm. (**M**) Day 9.5 of gestation. Inset: negative control. Scale bar = 800 μm. (**N**) Day 9.5 placenta. Scale bar = 100 μm. (**O**) Day 9.5 mesometrial decidua with dilated maternal vessels. Scale bar = 100 μm. (**P**) Day 10.5 of gestation. Inset: negative control. Scale bar = 800 μm. (**Q**) Higher magnification view of the placental labyrinth on day 10.5. Scale bar = 100 μm. (**R**) Dilated maternal vessels in the mesometrial decidua on day 10.5. Note the decrease in IGF1R immunoreactivity in the decidua at this stage. Scale bar = 100 μm. dec, decidua; e, embryo; epc, ectoplacental cone; fc, fetal capillary; gc, giant cell layer; jz, junctional zone; lab, labyrinthine zone; plac, placenta; ul, uterine lumen; mb, maternal blood space; *, dilated maternal blood vessel.


A
dec
B
dec
ul
C
e
D
ul
dec
e
E
epc
e
dec
F
dec
G
dec
e
H
ul
dec
I
dec
J
dec
plac
e
K
dec
ul
L
dec
gc
M
plac
dec
e
N
dec
jz
gc
lab
O
dec
P
plac
dec
e
Q
mb
fc
mb
mb
fc
R
dec

3.3.6 IGF2R protein expression

On days 6.5 and 7.5 of gestation IGF2R immunostaining is observed in the embryo and all trophoblast cells (Figure 3-6). On Day 8.5 there is strong immunostaining in the trophoblast cells of the EPC (Figure 3-6 H). However, this level of staining is decreased on day 9.5 in trophoblast cells (Figure 3-6 K) and by day 10.5 little to no IGF2R immunostaining is observed (Figure 3-6 N). Throughout early murine pregnancy, the decidua also stains positively for IGF2R, with more immunostaining around the dilated maternal vessels in the mesometrial decidua than in the anti-mesometrial decidua and that immediately surrounding the conceptus. This immunostaining is predominantly cytoplasmic, with staining also evident on the cell surface on days 9.5 and 10.5 (Figure 3-6 O). There also appears to be IGF2R immunostaining (presumably soluble IGF2R) in maternal blood in both the decidua and the placenta from day 7.5 onwards (Figure 3-6 F, N). No immunostaining is observed in blood in negative controls.

Figure 3-6 Localisation of IGF2R immunoreactvity in day 6.5 – 10.5 murine implantation sites (**A**) Day 6.5 of gestation. Scale bar = 800 μm. (**B**) Day 6.5 conceptus. Scale bar = 100 μm. (**C**) Cords of endothelial cells in the day 6.5 mesometrial decidua. Scale bar = 100 μm. (**D**) Day 7.5 implantation site. Scale bar = 800 μm. (**E**) Day 7.5 embryo and EPC. Scale bar = 400 μm. (**F**) The dilated maternal vessels of the day 7.5 mesometrial decidua. Note the immunostaining of soluble IGF2R in the maternal blood. Scale bar = 100 μm. (**G**) Day 8.5 of gestation. Inset: negative control. Scale bar = 800 μm. (**H**) The placenta and adjacent mesometrial decidua on day 8.5. Inset: negative control. Scale bar = 100 μm. (**I**) Day 8.5 mesometrial decidua. Scale bar = 100 μm. (**J**) Day 9.5 of gestation. Scale bar = 800 μm. (**K**) IGF2R immunostaining in the placenta on day 9.5. Scale bar = 100 μm. (**L**) Immunostaining for IGF2R in the mesometrial decidua surrounding the dilated maternal vessels. Scale bar = 100 μm. (**M**) Day 10.5 of gestation. Scale bar = 800 μm. (N) The placental labyrinth on day 10.5 of gestation. Note the IGF2R immunostaining in the maternal blood spaces. Scale bar = 100 μm. (**O**) Day 10.5 mesometrial decidua and its dilated maternal vessels. Scale bar = 100 μm. dec, decidua; e, embryo; epc, ectoplacental cone; fc, fetal capillary; gc, giant cell layer; jz, junctional zone; lab, labyrinthine zone; plac, placenta; ul, uterine lumen; mb, maternal blood space; *, dilated maternal blood vessel.


A
dec
e
B
e
gc
C
dec
D
epc
dec
e
E
*
ul
dec
epc
e
*
*
F
dec
*
*
*
G
dec
plac
e
H
dec
plac
I
dec
J
dec
plac
e
K
dec
jz
gc
lab
L
dec
*
*
M
dec
plac
e
N
fc
mb
O
*
dec
*
*

3.5 Discussion

This study provides the first series of photomicrographs demonstrating the pattern of cytokeratin labelled trophoblast invasion, as well as the changes in the decidual vasculature, and localization of IGF-II and the type 1 and 2 IGF receptors in mice from days 5.5 to 10.5 of gestation.

The pattern of trophoblast invasion is particularly interesting because, unlike in humans, and despite exhaustive serial sections, we found no evidence of murine TGCs in early to mid pregnancy invading the endometrium individually and up to day 10.5 of gestation they remained in close contact with the main giant cell layer. However, TGCs were found lying within endothelial lined maternal vessels at the leading edge of the main giant cell layer and all maternal blood spaces beneath this layer were lined by trophoblast. Due to their location, TGCs appear to play a direct role in displacing this endothelium, perhaps by phagocytosis, and therefore appear to play a role in the transformation from endothelium lined capillaries, into trophoblast lined maternal blood spaces (Adamson, Lu et al. 2002; Cross, Hemberger et al. 2002). Adamson et al. (2002) observed peri/endovascular TGCs 150-300 μm ahead of the main giant cell layer on day 12.5. Together these suggest that the initiation of TGC invasion away from the main giant cell layer occurs between days 10.5 and 12.5 of the 19 day gestation in mice. From day 12.5 of gestation TGCs are present only in and around the spiral arteries and are never seen individually. Conversely, the trophoblast glycogen cells that differentiate from spongiotrophoblast cells after day 12.5, and which can be identified by their vacuolated cytoplasm and strong IGF-II immunoreactivity and *Tpbp* gene expression, invade the decidua basalis in a diffuse interstitial pattern over the next three days of gestation (Redline, Chernicky et al. 1993; Teesalu, Blasi et al. 1998; Adamson, Lu et al. 2002). These invasive glycogen

cells have been considered to be homologous to human extravillous cytotrophoblasts (Redline, Chernicky et al. 1993) which undergo epithelial-mesenchymal transition to permit detachment from the extravillous cytotrophoblast cell columns and subsequent invasion of the decidua and its vasculature as individual cells (Vicovac and Aplin 1996) allowing extensive remodelling of the spiral arterioles.

Early in post-implantation pregnancy, the primary decidual zone (PDZ) displays a paucity of blood vessels that will supply the maternal contribution of the future placenta. Existing capillaries appear to undergo angiogenesis, the formation of new blood vessels as extensions of existing vessels, and there is an increase in both vessel number and diameter throughout early pregnancy. It is clear from the present study that remodelling of the decidual vasculature occurs early in post-implantation pregnancy. On day 5.5 the uterine arterioles in the outer undifferentiated stroma appear to be lined by smooth muscle, however those in the PDZ and in the non-decidualised stroma of the day 5.5 inter-implantation site do not. By day 8.5, many of the mesometrial vessels have become large diameter endothelial lined tubes and by day 10.5 of pregnancy the mesometrial, but not the anti-mesometrial, decidua has become a highly vascularised structure, with an extensive network of dilated vessels that presumably enable maximal blood supply to the developing placenta. Previous studies found that, by day 10.5 of pregnancy, murine decidual spiral arterioles are remodelled and lack a smooth muscle layer presumably due to its loss earlier in pregnancy (Adamson, Lu et al. 2002). However, the present study has shown that most of these vessels also lack a smooth muscle layer throughout early pregnancy. Therefore, these vessels never had a smooth muscle layer and are not strictly spiral arterioles.

It is important that transformation of the mesometrial vasculature occurs a significant distance from TGCs. Nevertheless, it has been proposed that TGCs affect decidual angiogenesis by secreting factors, most notably proliferin, that act on the distal endothelium. Placental proliferin expression is most abundant between days 8-10 of gestation (Linzer, Lee et al. 1985), is localised to TGCs (Lee, Talamantes et al. 1988; Jackson, Volpert et al. 1994) and stimulates endothelial migration in vitro and neovascularization *in vivo* (Jackson, Volpert et al. 1994). This process is dependent on proliferin binding to the promiscuous IGF2R (Volpert, Jackson et al. 1996) and is mediated through a G protein-coupled, mitogen-activated protein kinase-dependent pathway (Groskopf, Syu et al. 1997). We postulate that IGF-II, derived from the EPC and TGCs in early pregnancy may also play a role in initiating angiogenesis.

Alternatively, locally derived molecules may also act in the decidua to increase angiogenesis. Interestingly, we have found that IGF-II expression in the mesometrial decidua was strongest immediately adjacent to maternal blood vessels. IGF-II secreted by HepG2 human hepatocellular carcinoma cells has previously been shown to increase angiogenesis in a chick chorioallantoic membrane assay (Bae, Lee et al. 1998). Several studies have shown that IGF-II can induce the expression of the angiogenic factor vascular endothelial growth factor (VEGF) in immortalised HaCaT keratinocyte cells (Kwon, Kwon et al. 2004; Kim and Kim 2005). Consistent with this, it has been shown that VEGF is specifically expressed in the vicinity of proliferating endothelium in the murine reproductive tract (Shweiki, Itin et al. 1993). It was therefore suggested that VEGF forms a gradient of angiogenic activity directing the migration of endothelial cells towards the embryo. Consistent with this are the cords of endothelial cells apparently targeted to the conceptus. Therefore, we

suggest that IGF-II may act to increase VEGF synthesis in order to induce angiogenesis in the decidua.

The molecular mechanism by which IGF-II is likely to induce increased VEGF expression is currently under intense research and binding of IGF-II to either of its receptors is likely. It has been previously shown that the increase in angiogenesis by IGF-II was mediated by the IGF2R (Volpert, Jackson et al. 1996; Herr, Liang et al. 2003). In addition, impairment of IGF1R function significantly reduced VEGF expression in pancreatic tumour cells in vitro, and inhibited angiogenesis in vivo (Stoeltzing, Liu et al. 2003) suggesting that IGF-II may act via either IGF1R or IGF2R to increase VEGF induced angiogenesis.

We also found that IGF-II is expressed throughout early pregnancy in all layers of the trophoblast. These results were not surprising given that from day 9.5 in murine pregnancies, it has been shown that IGF-II is expressed strongly by the placenta (Redline, Chernicky et al. 1993) and in humans, IGF-II is expressed by the highly invasive extravillous cytotrophoblasts (Han, Bassett et al. 1996). IGF1R was strongly expressed in all trophoblast cells throughout early pregnancy. In contrast, expression of the IGF2R was highest earlier in pregnancy, when trophoblast proliferation and migration is most extensive, and declined by day 10.5 of gestation. This suggests that IGF-II may bind to either the IGF1R or to the IGF2R to promote placentation during these early stages of pregnancy. To date, there have been no reports examining the role of IGF1R mediated IGF-II action on trophoblast cells. However, in humans it has been shown that IGF-II, via binding to IGF2R, can induce a signal transduction cascade to promote invasion by an extravillous trophoblast cell line (McKinnon, Chakraborty et al. 2001). Also, in mice, IGF-II promotes TGC

transformation of EPC cells in culture and incubation with antibodies against IGF-II partially inhibit this process (Kanai-Azuma, Kanai et al. 1993). Therefore the role of IGF-II in placentation and the mechanism of its actions, including the receptor signalling pathways, warrant further elucidation.

In conclusion, there was no evidence of invasion by individual trophoblast cells nor of decidual vasculature remodelling by these trophoblast cells in early murine pregnancy. However, IGF-II and its receptors are localised to the trophoblast cells of the developing placenta, as well as the mesometrial decidua, supporting their role in both placentation and decidual angiogenesis. This suggests that, in mice, the trophoblast cells have less of a direct role in decidual vessel remodelling than is the case in humans, and may in fact secrete an array of molecules, including IGF-II, which can induce angiogenesis and vascular remodelling in the decidua.

Chapter 4
Complex Interactions between Hypoxia Inducible Factors, Insulin-Like Growth Factor-II and Oxygen in Early Murine Trophoblasts

4.1 Introduction

Hypoxia underlies a number of processes in which cellular migration and invasion occur including human placentation (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). In humans, endovascular cytotrophoblasts (CTBs) invade into and initially plug the uterine spiral arterioles, resulting in an oxygen tension within the interstitial space of below 18 mmHg (2.5%) at 8 weeks (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). The oxygen tension rises to approximately 60 mmHg (8.5%) at 12 weeks when the plugs are dislodged. CTBs replace the maternal endothelium and the spiral arterioles are remodelled dramatically increasing in diameter and are no longer responsive to vasoactive molcules in the maternal circulation. This increases blood flow to the placenta to provide for the demands of the growing fetus later in gestation (Zhou, Genbacev et al. 1998).

In mice, maternal blood in the placental labyrinth is not evident until day 10 of pregnancy (Muntener and Hsu 1977). To date, uterine oxygen tension during pregnancy in mice has not been measured but there is no evidence to suggest that trophoblasts ‘plug’ the spiral arterioles as is the case in humans. Also we have shown that murine trophoblast giant cells (TGCs) do not invade the decidua and mesometrium individually during the first half of pregnancy (see Chapter 2). Therefore, it is unknown whether oxygenated maternal blood reaches the conceptus in early gestation in mice.

Hypoxia Inducible Factors (HIFs) are a family of transcription factors that mediate the cellular response to hypoxia. HIFs form heterodimers consisting of one of three α subunits (HIF-1α, -2α, or -3α) bound to a β subunit (HIF-1β, -2β, or -3β) (Safran and

Kaelin 2003). In well-oxygenated cells, HIF-1α protein is rapidly degraded and has a half-life of less than 5 minutes (Wang, Jiang et al. 1995). However, under low oxygen conditions the α subunit is stabilised, allowing it to dimerise with the β subunit and bind to the hypoxia response element (HRE) in responsive genes (Rajakumar and Conrad 2000). Although it is widely believed that HIF-1α is not regulated at the mRNA level (Wenger, Rolfs et al. 1996), one study has shown that *Hif-1α* mRNA was upregulated by hypoxia (Wiener, Booth et al. 1996). HIFs are able to upregulate the expression of a range of genes including Vascular Endothelial Growth Factor (*Vegf*), Glucose Transporter-1 (*Slc2a1*) and Insulin-Like Growth Factor-II (*Igf2*) (Feldser, Agani et al. 1999; Wenger and Gassmann 1999; Safran and Kaelin 2003). Interestingly, IGF-II is important for fetal and placental development (DeChiara, Efstratiadis et al. 1990; Constancia, Hemberger et al. 2002; Sibley, Coan et al. 2004).

Both HIF-1 and HIF-2 are also essential for embryonic and placental development (Huang and Bunn 2003). *Hif1α* -/- mouse placentas exhibit impaired chorioallantoic fusion and vascularisation of the chorion. In contrast, *Hif-2α* -/- placentas have normal numbers of fetal blood vessels and proper invasion into maternal tissues (Cowden Dahl, Fryer et al. 2005). Impaired invasion of the placenta into the maternal decidua is further exacerbated in *Hif1α* -/- *Hif2α* -/- mice (Cowden Dahl, Fryer et al. 2005). Poorly invasive placentas also have decreased spongiotrophoblast cell numbers and expanded TGC numbers. Therefore, it appears that HIF-1 and HIF-2 regulate placental vascularisation and invasion, as well as trophoblast differentiation.

Currently, there is little information on the role of oxygen in murine placentation, the mechanisms by which the HIFs regulate placental invasion and differentiation nor their interaction with IGF-II in the placenta. Here we use immunohistochemistry to co-

localise regions of hypoxia and HIF-1α protein during early murine pregnancy. In addition, we cultured murine ectoplacental cones (EPCs) to determine the effect of, and interaction between oxygen and IGF-II on EPC outgrowth, trophoblast differentiation and the mRNA expression of HIFs and their target genes.

4.2 Materials and Methods

4.2.1 Mice

This study was approved by the Animal Ethics Committee of the University of Adelaide. C57BL/6 males and CBA/F1 females were obtained from the University of Adelaide Central Animal House, were housed on a cycle of 12 hours light, 12 hours dark and received food and water ad libitum. Two females were caged with each male overnight and checked for the presence of vaginal copulatory plugs the following morning (day 0.5 of pregnancy).

4.2.2 Pimonidazole Administration

To identify the cells that are hypoxic *in vivo*, mice were administered 60 mg/kg Pimonidazole (Hypoxyprobe-1; Chemicon International, Teneluca, CA) 2.5 hours prior to death via intra-peritoneal (i.p) injection. Pimonidazole is activated in an oxygen dependent manner and binds to thiol-containing proteins specifically found in hypoxic cells ($\leq$ 10 mmHg) (Varia, Calkins-Adams et al. 1998).

4.2.3 Immunohistochemistry

Histological analyses of implantation sites from each day of days 5.5 – 9.5 of development obtained from three different mice per day were performed. Mice were killed by cervical dislocation, uterine horns were removed and fixed in 4% paraformaldehyde overnight, before being divided into individual implantation sites.

Tissues were dehydrated in alcohol, embedded in paraffin blocks and cut into 6-μm serial sagittal sections.

Every tenth section was stained with haematoxylin and eosin (H&E) for general morphology. Additional sections were processed by immunohistochemistry to label Hypoxyprobe-1 (Chemicon International, Teneluca, CA, mAb, mouse anti-human pimonidazole, 1:25) or HIF-1α (Novus Biologicals, Littleton, CO, pAb, rabbit anti-human HIF-1α, 1:100, catalogue #NB-100-134). After deparaffinization and hydration, the sections were blocked for endogenous peroxidase activity (3% hydrogen peroxide for 30 minutes). To block for non-specific binding, sections were then treated with a serum free protein block (Dako Corporation, Carpinteria, USA) and incubated with the primary antibody at room temperature overnight. To block endogenous mouse immunoglobulins when using the Hypoxyprobe-1 mouse monoclonal antibody alternative blocking agents were applied prior to the primary antibody step following the manufacturer's instructions (BEAT Blocker Kit; Zymed, San Francisco, USA).

The sections were then incubated for 1 hour with their relevant secondary antibody. For Hypoxyprobe-1 goat anti-mouse IgG (1:500) and for HIF-1α, swine anti-rabbit IgG (1:400) were employed (Dako Corporation, Carpinteria, CA, USA). This was followed by 40 minutes incubation with horseradish peroxidase (HRP) conjugated with streptavidin (Rockland, Gilbertsville, PA, 1:500). The site of antibody binding was visualised with diaminobenzidine (DAB; Sigma, St Louis, USA) and counterstained with hematoxylin. The primary and secondary antibodies were diluted in a solution containing 1% bovine serum albumin (Sigma, St Louis, USA), 10% goat serum and

PBS. Negative controls used antibody diluent or irrelevant antibody in place of the primary antibodies.

4.2.4 Ectoplacental Cone and Decidua Dissection

Females were killed by cervical dislocation on day 7.5 of pregnancy. Uterine horns were excised and placed in Hank's Balanced Salt Solution (HBSS; Gibco, Grand Island, NY, USA), then transferred to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 with HEPES (DMEM/F12; Gibco, Grand Island, NY, USA) plus 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA) at 37°C. Implantation sites were separated and the myometrium removed. The embryo was then teased from the decidua and the EPC dissected from the remaining tissue (Nagy 2003).

4.2.5 Nuclear Protein Extraction and Nuclear HIF-1 Detection

Day 7.5 EPCs and deciduae were dissected as described above in HBSS containing a protease inhibitor (Complete, Mini, EDTA-free protease inhibitor cocktail tablets; Roche, Penzberg, Germany). Nuclear protein was then extracted using an Active Motif Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) with some modifications to the manufacturer's instructions for extracting from 3.2×10^6 cells. Briefly, the EPCs and deciduae were washed in PBS containing Phosphatase Inhibitors. The deciduae were minced with a scalpel blade, the EPCs and deciduae centrifuged for 5 minutes at 5.9 g at 4°C and the supernatant discarded. The pellet was then resuspended in 250 μl of 1x hypotonic buffer, mixed well and incubated on ice for 15 mins. The tissue was then digested in 25 μl of detergent, using the aid of a homogeniser for the decidua. This was then centrifuged for 30 secs at 13.4 g at 4°C and the supernatant (cytoplasmic fraction) discarded. The pellet was then resuspended in 25 μl lysis buffer, incubated on ice for 30 mins and centrifuged for 10 mins at 13.4 g at 4°C. The

supernatant (nuclear fraction) was then transferred to a fresh tube and stored at -80°C.

EPCs from 2 mice were pooled giving a total 12-15 EPCs per sample (N=6) and 28 deciduae from 12 mothers. EPC samples were diluted 1:50 and deciduae samples diluted 1:200 in water and a RC DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) was performed to quantify the nuclear protein in each sample.

To quantify the amount of nuclear HIF-1 protein in the EPC and decidual samples, a TransAm HIF-1 kit (Active Motif, Carlsbad, CA, USA) was used according to the manufacturer's protocol. This kit contains a 96-well plate precoated with an oligonucleotide containing the hypoxia response element (HRE; 5'-TACGTGCT-3') from the *EPO* gene. HIF dimers contained in the nuclear extracts bind specifically to this oligonucleotide and are detected via an antibody directed against an epitope on HIF-1α that is accessible upon DNA binding. The secondary antibody is HRP-conjugated and provides a sensitive colorimectric readout that can be quantified by spectrophotometry. Cos-7 nuclear extract provided with the kit was used as a positive control. All samples and positive controls contained 5 μg of nuclear protein per well.

4.2.6 Ectoplacental Cone Cultures

EPCs were distributed such that those from the same mother were divided among the treatment groups. 2-3 EPCs per well were cultured in DMEM containing 10% FCS or on Matrigel coated wells to determine the effects of the culture conditions on EPC outgrowth. For all subsequent experiments, EPCs were cultured in serum free media on Matrigel. Briefly, Nunclon 4-well plates were coated with 20 μl of Growth Factor Reduced BD Matrigel Matrix (BD Biosciences, Bedford, MA, USA) and left to

set for 30 minutes at 37°C. 2-3 EPCs per well were cultured in DMEM containing 2% Nutridoma-SP (Roche Diagnostics, Mannheim, Germany), 20 ng/ml plasminogen (Sigma-Aldrich Pty. Ltd., St. Louis, MO, USA) and 0.04 mM L-Glutamine (Invitrogen, Carlsbad, CA, USA) for 3 days at 37°C in 20%, 5% or 1% oxygen, with or without 125 ng/ml IGF-II (GroPep Ltd, Thebarton, SA, Australia). Culture plates were placed in modular incubator chambers (Billups Rothenburg, Del Mar, CA) and flushed for 5 minutes at 5 L/min with 20%, 5% or 1% oxygen gas mixtures containing 5% CO_2 balanced with N_2. All media used were equilibrated overnight in the correct oxygen tension prior to use and the oxygen tension in the modular chambers was measured with an oxygen analyser (Teledyne Instruments, City of Industry, CA, USA) regularly. EPCs were digitally photographed as soon as they were placed into the culture wells and at the end of the culture period. The area of trophoblast outgrowth was measured using VideoPro Video Image Analysis software (Leading Edge, Marion, SA).

4.2.7 RNA Extraction and Reverse Transcription

RNA was extracted from wells containing pairs of day 3 cultured EPCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 1 ml TRIzol was added to each well and incubated for 5 mins at room temperature before adding 200 μl chloroform. Each tube was then shaken for 15 seconds, incubated for 3 mins and centrifuged for 15 mins at 12,000g at 4°C to separate the RNA from the other cellular components. The top aqueous layer was removed and put into a new tube. 500 μl isopropanol and 20 μg glycogen were added to each tube before incubating overnight at -80°C. The following day, the samples were centrifuged for 25 mins at 12,000g at 4°C and the supernatant removed. Each tube had 300 μl of 70% ethanol added and was then centrifuged for

20 mins at 12,000g at 4°C. The supernatant was removed and the pellet left to air dry at room temperature for 10 mins. Final RNA pellets were redissolved in 10 μl sterile water and samples were DNAse treated (DNA-free; Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions to eliminate contaminating genomic DNA.

RNA (1 μg) from each sample was reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). RNA from an *in vivo* developed day 14 mouse placenta was used to generate a pooled standard cDNA. 200 ng random hexamer primers was added to the RNA and incubated at 65°C for 10 min, followed by 3 min incubation on ice. Reverse transcription was then performed in a final volume of 40 μl, containing buffer (1x), 10 mM DTT, 1 mM of dNTPs and 2 μl SuperScript III Reverse Transcriptase. Reactions were incubated for 5 min at 25°C, followed by 45 min at 50°C then 70°C for 15 min. Additional controls included no RT controls (-RT) using day 14 placental RNA with no reverse transcriptase and non-template controls (NTC) which had no RNA.

4.2.8 Real Time Quantitative PCR

Real-time PCR was performed in an Applied Biosystems 7000 GeneAmp Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were designed over exon-exon boundaries, where possible, using Primer Express (Applied Biosystems, Foster City, CA, USA) and were synthesised by Sigma Genosys (Sigma Genosys, Sydney, Australia). Primer sequence details are described in Table 4-1. Sample cDNA was diluted in water to a concentration per microliter equivalent to cDNA generated from 0.25 ng starting RNA. PCR was performed in 10 μl volumes containing 5μl SYBR Green Master Mix (Applied

Biosystems, Foster City, CA, USA) and 2.5 μl diluted cDNA (0.625 ng equivalent). Primers were diluted to 1.25 μM for *18s* rRNA, Insulin-Like Growth Factor -II (*Igf2*) and the Type 2 IGF Receptor (*Igf2r*), 2.5 μM for Hypoxia-Inducible Factor-1α (*Hif-1α*), *Hif-2α* antisense HIF-1α (*asHif-1α*), Vascular Endothelial Growth Factor (*Vegf*), Glucose Transporter 1 (*Slc2a1*) and Placental Lactogen-1 (*Pl-1*), and 5 μM for *Tpbp* (also known as *Tpbpa* or *4311*) and *Tfeb*, and 0.5 μl of each primer was added to each reaction. The thermal cycling program consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 60°C. A cycle threshold (Ct) was calculated for each sample using the GeneAmp 7000 software. Dissociation curves, to detect non-specific amplification, were generated for all reactions, and negative control samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification products. The PCR products were analysed on 5% agarose gel containing ethidium bromide in TAE 1x and photographed (data not shown). The standard curve method of quantification was used and all samples were normalised to the housekeeping gene (*18s*).

Table 4-1 Primer sequences used for real time RT-PCR

Gene	Genbank Accession number	Primer sequence	Amplicon size (bp)
18S	AF176811	Forward - AGAAACGGCTACCACATCCAA Reverse - CCTGTATTGTTATTTTTCGTCACTACCT	91
Hif-1α	NM_010431	Forward - TCAGAGGAAGCGAAAAATGGA Reverse - AGTCACCTGGTTGCTGCAATAAT	77
Hif-2α	BC057870	Forward - CTATGTGCTGAGTGAGATCGAGAAG Reverse - GATTCGGTCTGGTCCATGGA	60
asHif-1α	AY279360	Forward - GAGAGCGCGGAAAACTCTTGT Reverse - CATTAGCAGGTGAAGGAAGCTAGG	51
Vegfa	NM_009505	Forward - CTGCTGTGCTGTAGGAAGCTCAT Reverse - CCACGTCAGAGAGCAACATCA	75
Glut-1	M23384	Forward - CCAGCTGGGAATCGTCGTT Reverse - CAAGTCTGCATTGCCCATGAT	76
Igf2	M14951	Forward - CGGCCCCGGAGAGACT Reverse - GGTTGGCACGGCTTGAAG	51
Igf2r	NM_010515	Forward - ATCACTCCTGGAATTCAACACCA Reverse - TGTGAATCTGAAGGCTGGCA	51
Tfeb	AF079095	Forward - GTCTAGCAGCCACCTGAACGT Reverse - ACCATGGAGGCTGTGACCTG	51
Tpbp	NM_009411	Forward - GCCAGTTGTTGATGACCCTGA Reverse - CCCATCGCCACTCTCTGTGT	51
Pl-1	M35662	Forward - GTCTTGAGGTGCCGAGTTGTC Reverse - CTGGGTGGGCACTCAACATT	51

4.2.9 Statistics

All data are presented as means ± SEM. All statistical analyses were carried out using SPSS Version 13 for Windows (SPSS, Chicago). All comparisons between treatment groups were performed using Mann Whitney U test.

4.3 Results

4.3.1 Hypoxia and HIF-1α localisation in early murine implantation sites

To determine which cells in the implantation site were exposed to hypoxia, pregnant mice were injected with Hypoxyprobe-1 and immunohistochemistry was employed to localise pimonidazole adducts (Figure 4-1). On days 5.5-7.5, decidual cells surrounding the apoptosed uterine lumen were labelled, with the greatest area of staining on day 6.5. There was no labelling of trophoblast cells or the yolk sac at any stage, and only weak staining of the embryo on day 6.5. On days 8.5 and 9.5 there

was no labelling anywhere in the implantation site, neither in the decidua nor conceptus.

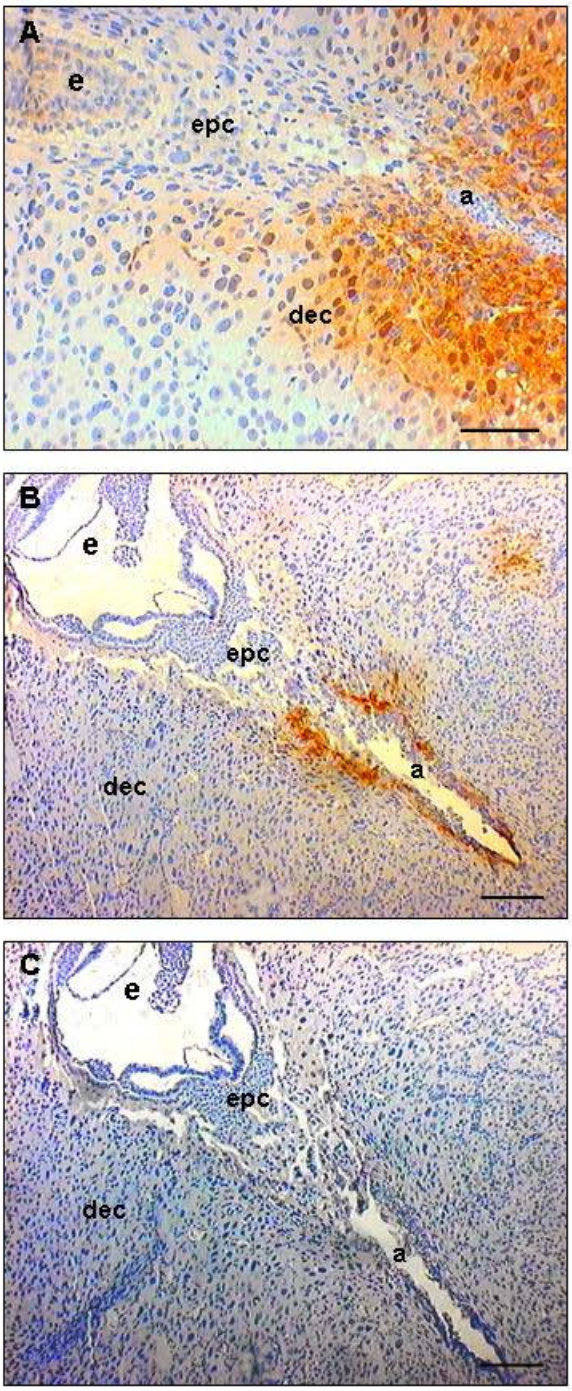


Figure 4-1 Localisation of hypoxic regions via pimonidazole binding in day 6.5-7.5 murine implantation sites
(**A**) At day 6.5 of gestation intense staining surrounds the apoptotic luminal epithelium. Scale bar = 25 μm. (**B**) At day 7.5 of gestation the area of immunostaining of Hypoyprobe-1 was reduced. Scale bar = 200 μm. (**C**) Day 7.5 negative control, scale bar = 200 μm. a, apoptotic uterine luminal epithelium; e, embryo; epc, ectoplacental cone; dec, decidua.

We next wanted to assess the localisation of HIF-1α and determine whether it was colocalised with hypoxyprobe (Figure 4-2). On day 5.5, there was weak cytoplasmic HIF-1α staining in the trophoblast, embryo and decidua surrounding the conceptus. However, on day 6.5, HIF-1α displayed strong cytoplasmic and nuclear localisation in most areas of the implantation site including the embryo, EPC, entire decidua and in the outer myometrium of the uterus. Similar staining was observed on day 7.5 in the EPC and decidua. On day 8.5, HIF-1α protein was still strongly expressed in the mesometrial decidua but was no longer immunoreactive in the embryo or the antimesometrial decidua and staining in the EPC was relatively weak. On day 9.5, HIF-1α was localised to the trophoblasts of the labyrinthine zone, especially those lining the maternal blood spaces, and in the cytoplasm of the TGCs. HIF-1α localisation in the decidua was similar to that observed in earlier pregnancy.

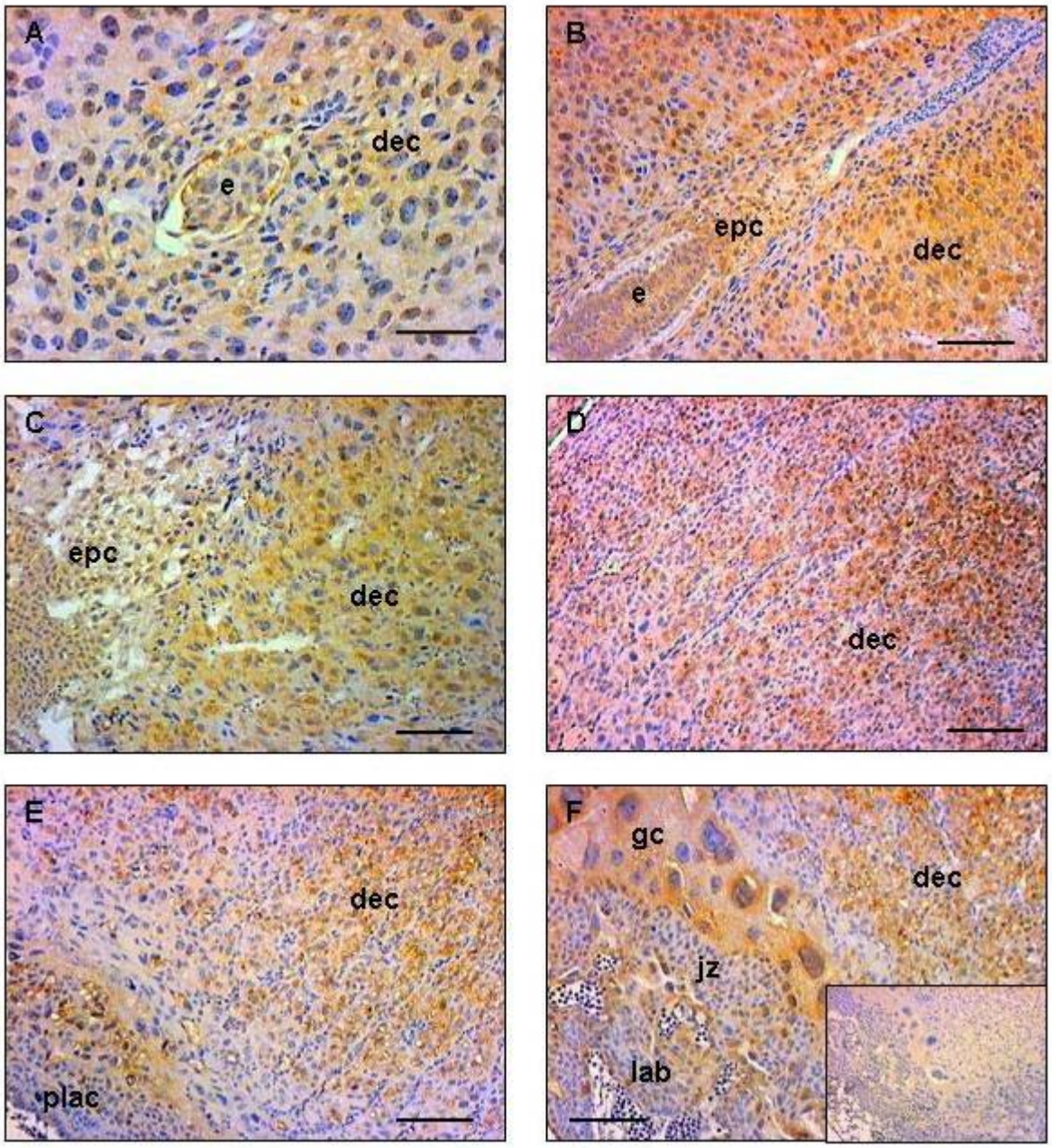


Figure 4-2 Localisation of HIF-1α on day 5.5-9.5 by immunohistochemistry
(**A**) HIF-1α is present in the decidua and the conceptus on day 5.5. Scale bar = 50μm. (**B**) At day 6.5 both nuclear and cytoplasmic HIF-1α immunostaining is apparent throughout the implantation site. (**C**) At day 7.5 there is immunostaining in the EPC and decidua. (**D**) At day 7.5 intense nuclear and cytoplasmic HIF-1α labelling was present in the mesometrial decidual cells surrounding the intricate network of blood vessels. (**E**) At day 8.5 HIF-1α immunostaining is apparent in the decidua and in trophoblasts at the maternal border of the placenta. (**F**) Day 9.5 showing HIF-1α labelling in the TGCs and in the newly developing labyrinth. Inset: negative control. e, embryo; epc, ectoplacental cone; dec, decidua; gc, trophoblast giant cell layer; jz, junctional zone; lab, labyrinth (exchange region); plac, placenta. Scale bars (**B-F**) = 200μm.

4.3.2 Quantification of Nuclear HIF-1 Protein in EPC and Decidua

Day 7.5 *in vivo* derived EPC and deciduae were extracted and assessed for nuclear HIF-1 protein levels. The positive control sample that consisted of $CoCl_2$ - treated COS-7 cell nuclear protein strongly expressed HIF-1α (data not shown). In addition, HIF-1α protein was present in nuclear extracts of both the EPC and the maternal decidua on day 7.5, and was more abundant in the EPC than in the decidual cells (Figure 4-3).

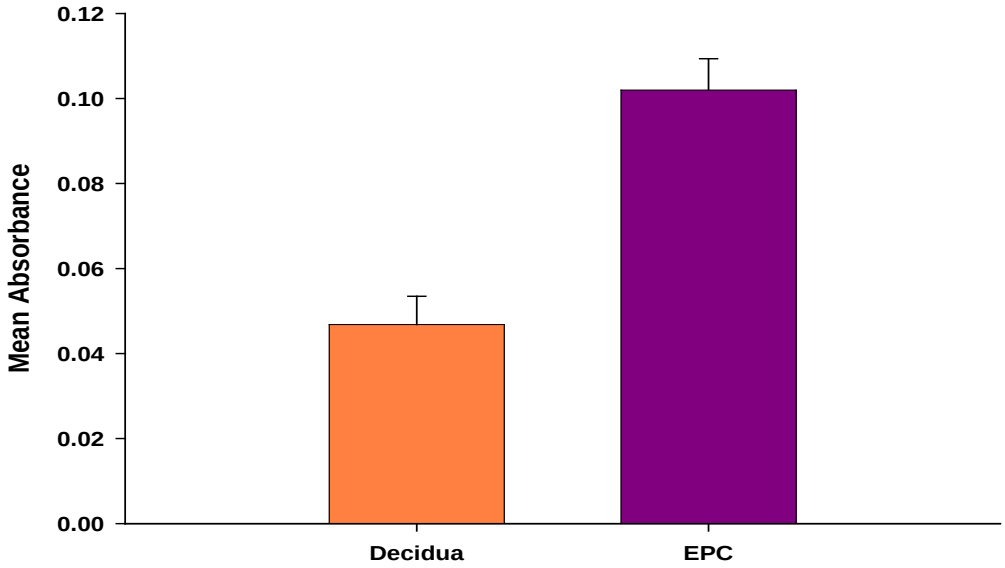


Figure 4-3 Expression of HIF-1 nuclear protein in the EPC and decidua on day 7.5
Data are expressed as mean absorbance ± SEM. Nuclear HIF-1 protein was present in the EPC and decidua on day 7.5.

4.3.3 Effect of Serum and Matrigel on EPC outgrowth

Firstly, day 7.5 EPCs were cultured for 3 days in varying oxygen concentrations in either media containing 10% FCS, or in serum free media on Matrigel coated wells. When cultured in 20% oxygen there was no difference between those cultured in FCS or on Matrigel on the area of EPC outgrowth. However, when cultured in a low oxygen environment (5% or 1% oxygen), culture on Matrigel significantly reduced the area of EPC outgrowth by 39% and 36% respectively, compared to those cultured in the presence of FCS ($p<0.005$). Therefore, the culture environment had a significant

effect on the response to the oxygen concentration (p=0.001). For subsequent experiments looking at the effects of, and interactions between, oxygen and IGF-II, EPCs were cultured in serum free media on Matrigel coated wells as we believed this better mimicked the *in vivo* environment.

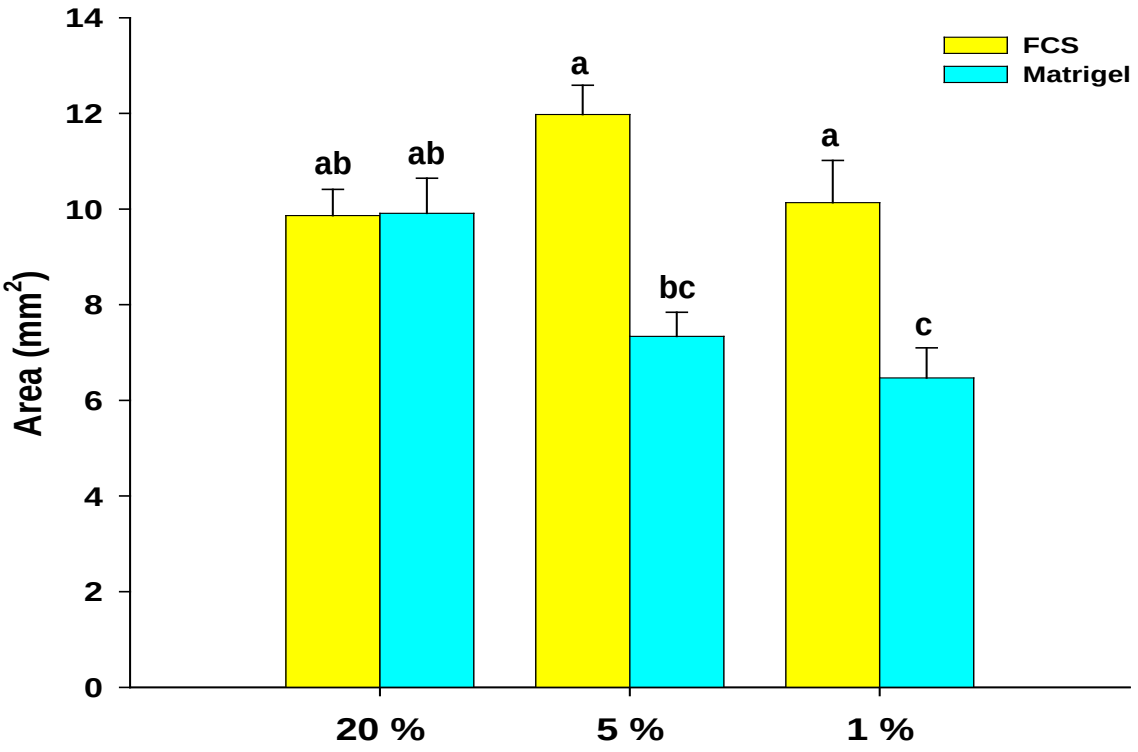


Figure 4-4 Effect of culture conditions on EPC outgrowth
Data are expressed as mean ± SEM. Different superscripts denote differences between groups, p<0.005. Effect of oxygen on Matrigel with no FCS, p<0.05. Effect of substrate, with or without FCS, p<0.001. Combination effect of oxygen and substrate, p=0.001.

4.3.4 Effect of Oxygen and IGF-II on EPC Outgrowth

EPCs throughout the culture period were photographed and the area of outgrowth was measured (Figure 4-5). Although not significant, there was a 17% decrease in the area of EPC outgrowth when cultured in 5% oxygen compared to those cultured in 20% oxygen. However, culture in 1% oxygen significantly decreased the area of EPC outgrowth by 34.7% (p<0.01). The addition of 125 ng/ml exogenous IGF-II had no effect on EPC outgrowth (Figure 4-5).

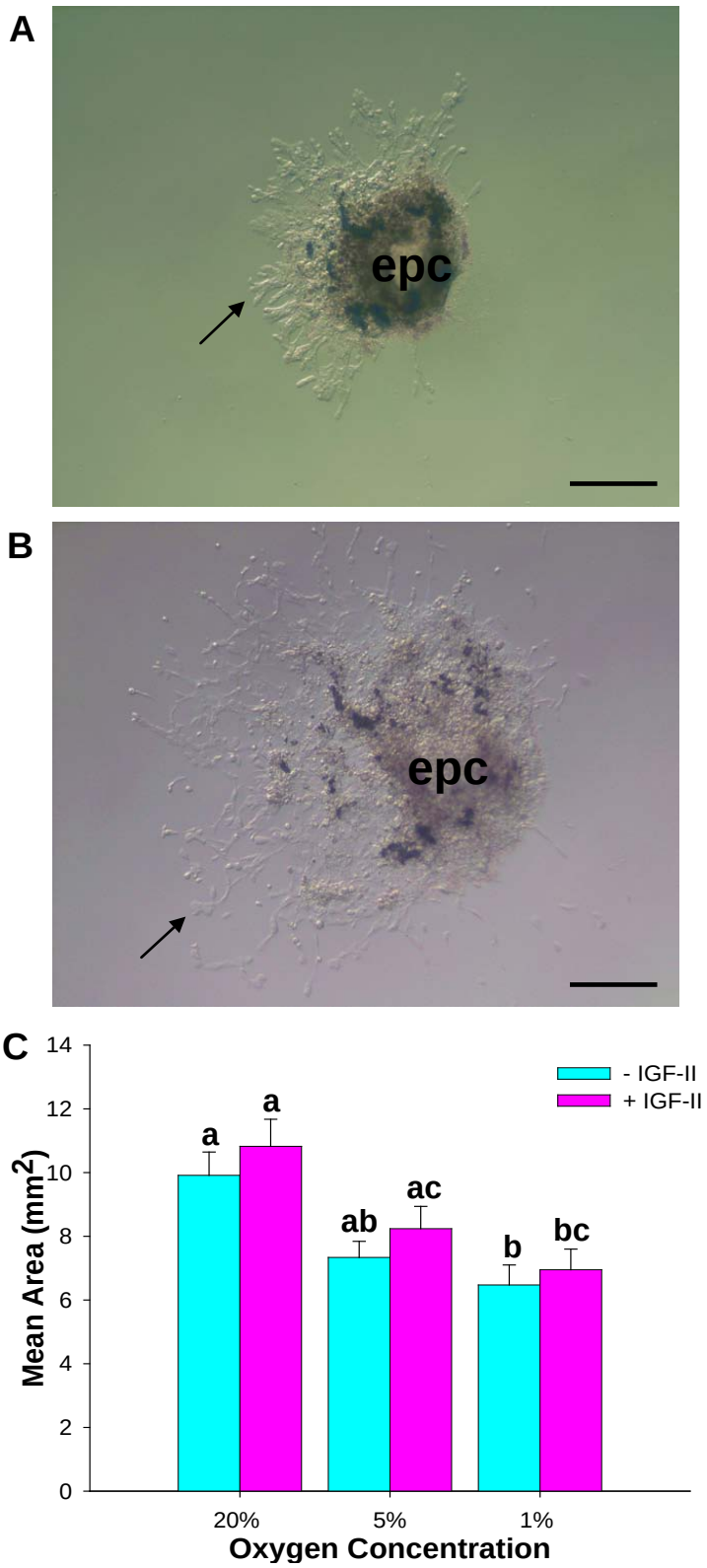


Figure 4-5 Effect of oxygen and exogenous IGF-II on EPC outgrowth
(**A**) A representative EPC cultured in 20% oxygen with the addition of IGF-II photographed after 24h of culture. (**B**) The same EPC as in (**A**) after 3 days in culture. (**C**) Area of EPC outgrowth. Data are shown as mean ± SEM, N=42-48 per treatment group. Different superscripts denote differences between groups, $p<0.05$. Scale bars = 250μm. Arrow, migrating trophoblast cells; EPC, ectoplacental cone.

4.3.5 Effect of Oxygen and IGF-II on Markers of Differentiation in EPCs

To determine whether oxygen and/or IGF-II alter the trophoblast lineage to which EPC cells commit, *Tfeb* gene expression was used as a marker of syncytiotrophoblast, *Tpbp*, a marker of spongiotrophoblast and *Pl-1* a marker of TGCs. *Tfeb* and *Pl-1* mRNA levels were unaffected by oxygen concentration. However, the levels of *Tpbp* mRNA were significantly increased by 124% and 387% in 1% cultures compared to those cultured in 5% and 20% oxygen respectively ($p=0.04$ and $p=0.007$, respectively). The addition of exogenous IGF-II had no effect on the levels of *Tfeb*, *Pl-1* or *Tpbp* mRNA (Figure 4-6).

4.3.6 Effect of Oxygen and IGF-II on *Hif* mRNA in EPCs

Hif-1α and *Hif-2*α mRNA levels were measured to determine if HIF gene expression in EPC cultures changed in response to oxygen concentration (Figure 4-7). *Hif-1*α mRNA levels were significantly decreased by 37% and 106% when cultured in 5% and 1% oxygen, respectively ($p=0.02$ and $p=0.002$) compared to 20% cultures. However, IGF-II treatment had no effect on *Hif-1*α mRNA levels. Conversely, oxygen concentration had no effect on *Hif-2*α mRNA levels, whilst the addition of exogenous IGF-II significantly reduced the level of *Hif-2*α mRNA when cultured in 1% oxygen but not in 20% or 5%.

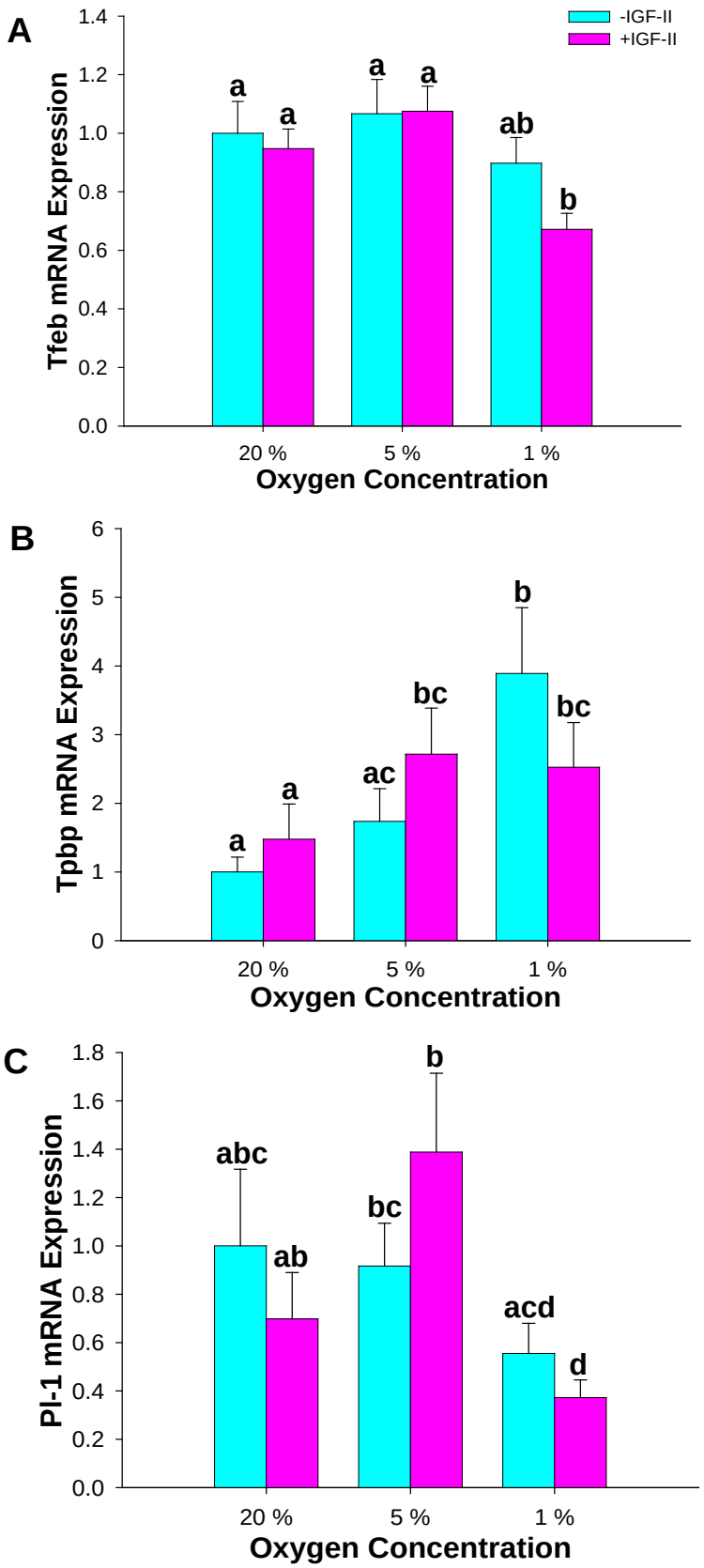


Figure 4-6 Effect of oxygen and exogenous IGF-II on markers of trophoblast differentiation
(**A**) *Tfeb* mRNA expression in EPCs. (**B**) *Tpbp* mRNA expression. (**C**) *Pl-1* mRNA expression. Results were normalized to *18s* and are expressed relative to the 20% - IGF-II group. Data are shown as mean ± SEM, N=13-20 per treatment group. Different superscripts denote differences between groups, $p<0.05$.

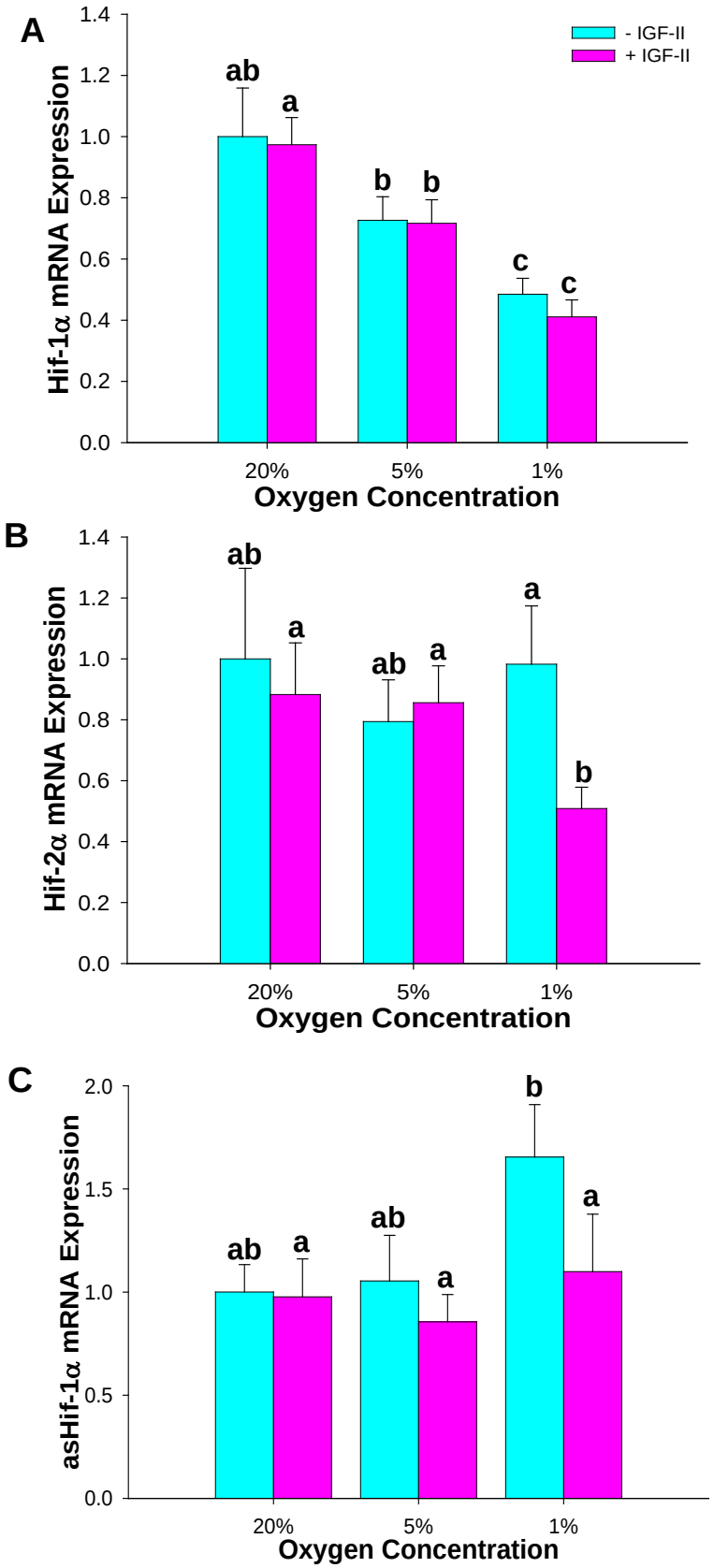


Figure 4-7 Effect of oxygen and IGF-II on *Hif* mRNA levels
(**A**) Hif-1α mRNA expression in EPCs. (**B**) Hif-2α mRNA expression. (**C**) asHif-1α mRNA expression. Results were normalized to 18s and are expressed relative to the 20% - IGF-II group. Data are shown as mean ± SEM, N=13-21 per treatment group. Different superscripts denote differences between groups, $p<0.05$.

Since naturally occurring antisense HIF-1α (asHIF-1α) is a negative regulator of *Hif-1α* mRNA stability, we hypothesised that *asHif-1α* levels may be increased by the low oxygen environment and that this may contribute to the decrease in *Hif-1α* mRNA seen following culture in 1% oxygen. Culture in 1% oxygen tended to increase *asHif-1α* mRNA levels compared to those cultured in 20% oxygen (p=0.08) and 5% oxygen (p=0.06). *asHif-1α* was significantly different in the 1% oxygen group compared to the 20% and 5% oxygen groups when IGF-II was added (p=0.03 and p=0.02, respectively). However, treatment with IGF-II at 1% oxygen abolished the effect of low oxygen conditions on *asHif-1α* concentration (Figure 4-7).

The HIF target genes *Vegf* and *Slc2a1* are both essential in placental development. Therefore we wished to determine if the changes in *Hif* mRNA levels affected the expression of HIF target genes (Figure 4-8). Culture in 5% oxygen significantly reduced *Vegf* mRNA levels compared to 20% and 1% oxygen. Addition of exogenous IGF-II reduced V*egf* mRNA by 34% at 1% oxygen, but not at 20% or 5% oxygen (p=0.006). In contrast, culture in decreasing oxygen concentrations significantly increased *Slc2a1* mRNA levels in a dose dependent manner resulting in over a two-fold increase in the expression of *Slc2a1* at 1% oxygen, compared to 20% oxygen (p<0.001). Treatment with IGF-II reduced *Slc2a1* mRNA levels at 1% oxygen, but not at 20% or 5% oxygen.

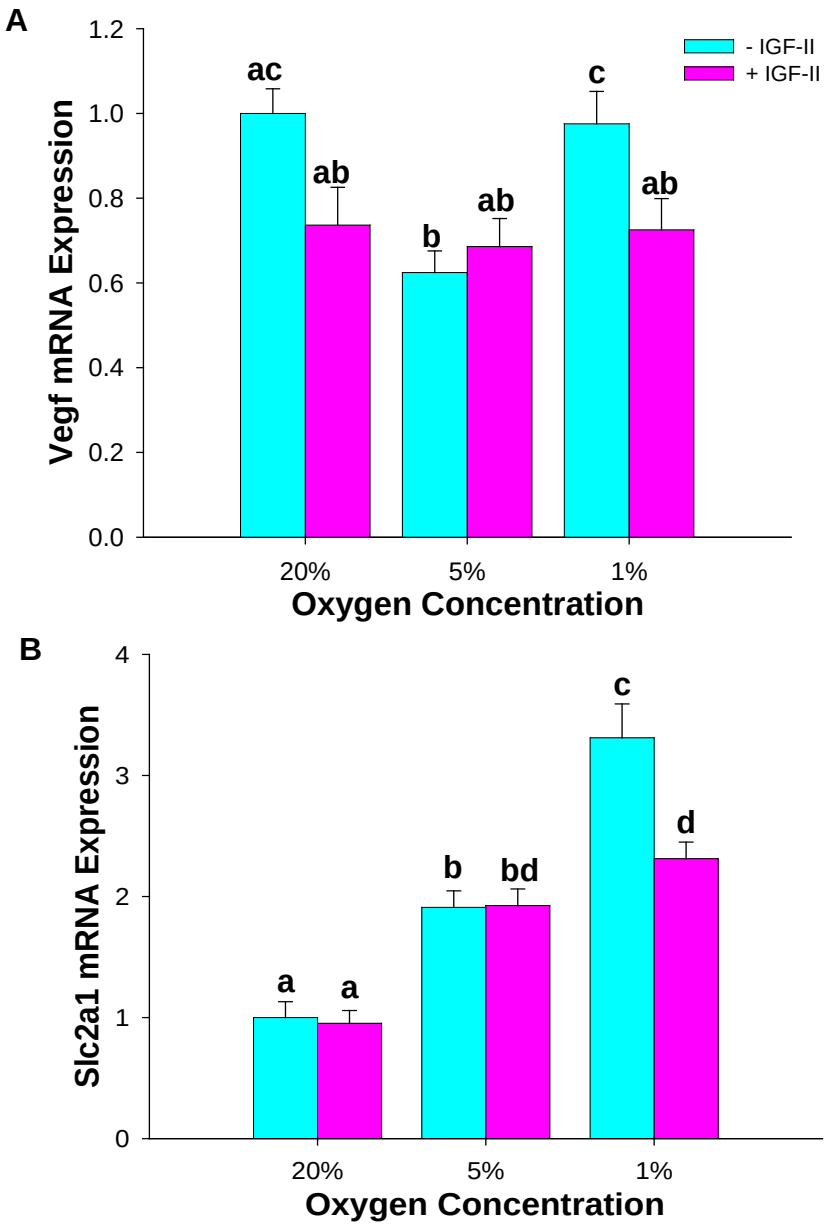


Figure 4-8 Effect of oxygen and IGF-II on HIF target genes, *Vegf* and *Slc2a1*
(**A**) *Vegf* mRNA expression in EPCs. (**B**) *Slc2a1* mRNA expression. Results were normalized to 18s and are expressed relative to the 20% - IGF-II group. Data are shown as means ± SEM, N=15-20 per treatment group. Different superscripts denote differences between groups, $p<0.05$.

4.3.7 Effect of Oxygen and IGF-II on *Igf2* and *Igf2r* mRNA in EPCs

Since IGF-II is also itself a HIF target gene and has the potential to regulate its own expression, we determined whether oxygen or exogenous IGF-II affect endogenous *Igf2* mRNA levels in the cultured EPCs (Figure 4-9). *Igf2* mRNA was significantly reduced by culture in 1% oxygen ($p<0.05$) compared to culture in 5% or 20% oxygen.

However, the addition of exogenous IGF-II had no effect on endogenous *Igf2* mRNA expression in EPCs. Neither oxygen nor treatment with exogenous IGF-II had any effect on *Igf2r* mRNA levels in cultured EPCs.

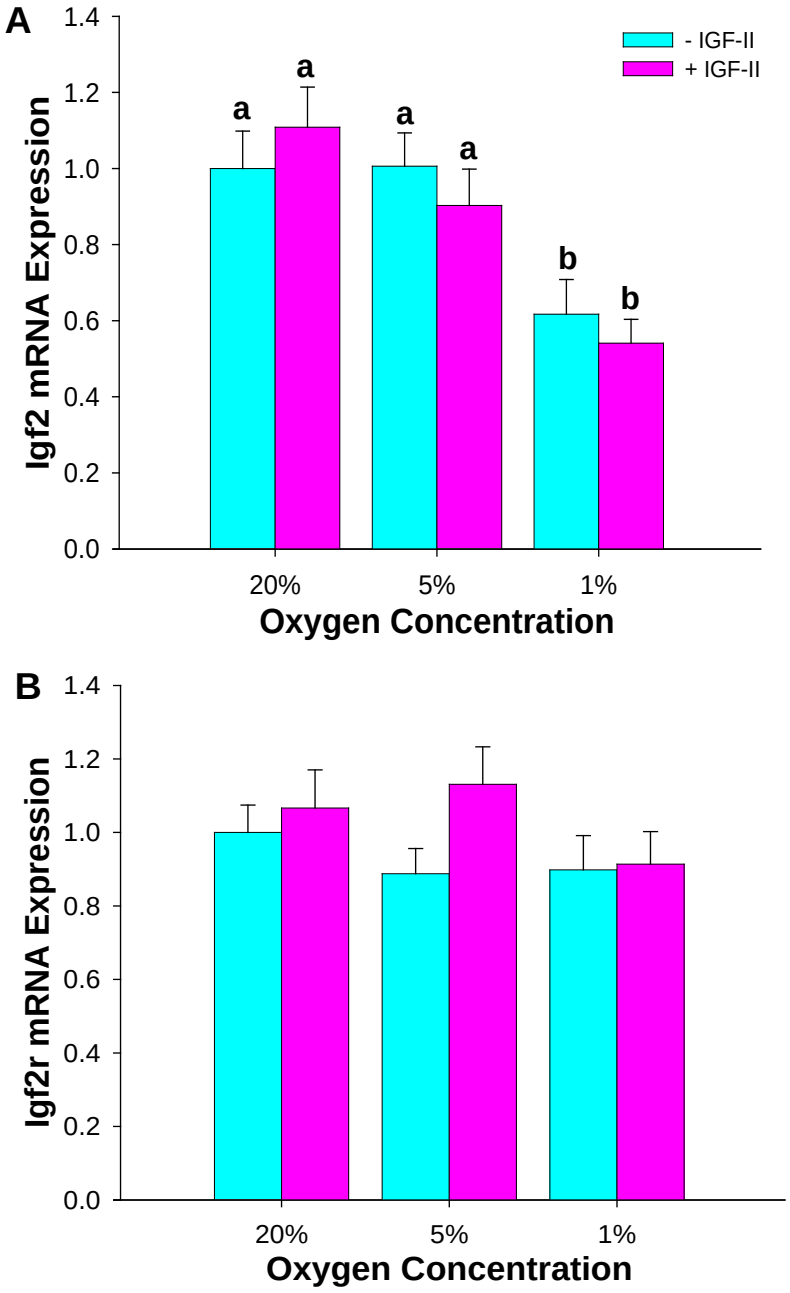


Figure 4-9 Effect of oxygen and exogenous IGF-II on endogenous *Igf2* and *Igf2r* mRNA levels
(**A**) *Igf2* mRNA expression in EPCs. (**B**) *Igf2r* mRNA expression. Results were normalized to *18s* and are expressed relative to the 20% - IGF-II group. Data are shown as mean ± SEM, N=15-21 per treatment group. Different superscripts denote differences between groups, $p<0.05$.

4.4 Discussion

We have shown that the early murine implantation site is exposed to oxygen tensions lower than 1.5% oxygen on day 6.5 - 7.5. *In vitro*, in 1% oxygen, EPC outgrowth was reduced and the expression of *Tpbp* was increased, suggestive of trophoblast differentiation to the spongiotrophoblast lineage. Our data also indicate that *Hif-1*α and *Hif-2*α mRNA abundance is differentially regulated by oxygen and IGF-II and that *asHif-1*α may be a mediator of the response to prolonged hypoxia in murine trophoblasts *in vitro*.

Our data suggest that the early murine conceptus is exposed to oxygen levels similar to those detected in early human placentae (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). The only localisation of pimonidazole in the mouse implantation site was on days 6.5 and 7.5. However, pimonidazole only detects tissues exposed to oxygen levels of $\leq$10 mmHg (1.5% oxygen) and hence would not detect concentrations of oxygen consistent with milder hypoxia. It is likely then that the regions surrounding the pimonidazole labelling may also be experiencing a relatively low oxygen environment. On day 6.5 there is extravasated blood in the EPC (Muntener and Hsu 1977), possibly due to the invasion of the TGC at this time and the destruction of the endothelium as the embryo implants, and little vascularisation of the decidua (see Chapter 3). Hence, the surrounding vessels may not be intact. However, the yolk sac provides oxygen and nutrients to the embryo during these early stages. We hypothesise that there is an oxygen gradient in the implantation site during these early stages of pregnancy and, as the vessels in the decidua undergo angiogenesis and dilate, the blood supply to the conceptus gradually increases.

It is known that in early murine pregnancy HIF is regulated by a combination of factors, including oxygen, growth factors and hormones. Importantly, it was previously shown that in the placenta, HIF-α induction can occur independent of oxygen tension (Maltepe, Krampitz et al. 2005). This study has found nuclear HIF-1α protein in both the EPC and the decidua on day 7.5 of pregnancy *in vivo*. In addition, HIF-1α was localised in the decidua later in pregnancy when no regions of hypoxia were identified using the hypoxyprobe marker. Similarly, HIF-1α protein and mRNA were previously detected in the decidua on day 7.5 of pregnancy (Daikoku, Matsumoto et al. 2003). In contrast to the current study, a previous report found that there was no HIF-1α protein detected in the EPC on day 8.5 (Schaffer, Vogel et al. 2005). However, we did see reduced placental HIF-1α expression on this day, with staining only expressed at the fetomaternal border of the placenta. However, on day 9.5, there was strong expression of HIF-1α in the TGC layer and in the newly developed labyrinth. In the future it will be important to also determine the localisation of HIF-2α.

Current understanding of the influence of oxygen on human trophoblast migration and invasion is somewhat confusing. Some studies suggest that culture of a human first trimester extravillous cytotrophoblast cell line in low (1%) oxygen conditions increases invasion of these cells when compared to culture in 20% oxygen (Graham, Fitzpatrick et al. 1998). In contrast, culture of HTR-8/SVneo cells in 2% oxygen has been shown to decrease trophoblast invasion and increase cell proliferation (Kilburn, Wang et al. 2000). Similarly, other studies found that culturing first trimester villous explants in low oxygen (2 or 3% oxygen) stimulates proliferation of CTBs in cell columns (Genbacev, Joslin et al. 1996; Genbacev, Zhou et al. 1997; Caniggia, Mostachfi et al. 2000). In disagreement with these reports is a recent study where

culture of 8-12 week human placental villous explants in 1.5% oxygen resulted in fewer explants producing EVT outgrowths and a decreased area of outgrowth and number of cells emanating from these explants (James, Stone et al. 2006). There are several possibilities as to why these findings are so disparate. It may be due to the differences in the populations of cells used, for example isolated CTBs compared to villous explants, the gestational age from which the cells were derived or the presence or absence of serum and hence factors which may have either an inhibitory or stimulatory effect on trophoblast invasion.

To date, there are no written reports on the role of oxygen on murine trophoblast migration *in vitro*. However, culture of rat Rcho-1 cells in 1% oxygen inhibited their differentiation to TGCs, and maintained their stem cell-like state (Gultice, Selesniemi et al. 2006). Here, we demonstrate that mouse trophoblast cells respond differently to oxygen depending on whether they are cultured with serum on plastic, or in serum free media on Matrigel coated wells but the latter is more likely to mimic the *in vivo* environment. Therefore care needs to be taken when analysing any *in vitro* data. We determined that exposure of day 7.5 murine EPCs to 1% oxygen for a prolonged period (3 days) inhibits EPC outgrowth when cultured on Matrigel. This treatment may induce a stress response in these trophoblasts since *in vivo* we could detect no hypoxyprobe labelling in EPCs on day 7.5. Therefore it seems likely that the 1% oxygen *in vitro* conditions were lower than those experienced *in vivo* at that time.

The decrease in EPC outgrowth observed under low oxygen conditions may be a result of a decrease in cell proliferation or reflect a change in the differentiation state of the trophoblasts, rather than a decrease in migration *per se*. Indeed, it was recently proposed that upregulation of the HIF complex in low oxygen promotes

formation and maintenance of the spongiotrophoblast lineage (Adelman, Gertsenstein et al. 2000; Cowden Dahl, Fryer et al. 2005). In the current study, culture in low oxygen significantly increased the mRNA expression of the spongiotrophoblast marker *Tpbp*, indicating that oxygen may indeed affect trophoblast differentiation in the early mouse placenta. This is consistent with the findings of Cowden-Dahl and colleagues and it is possible that this may be in part HIF-2α mediated, since the expression of *Hif-1α,* but not *Hif-2α*, mRNA was reduced in EPCs cultured in low oxygen. In addition, this cell lineage would presumably be less invasive than, for example, a population of TGCs, and may account for our observed decrease in EPC outgrowth in the low oxygen environment.

Since *Igf2* is a HIF target gene (Feldser, Agani et al. 1999) and was shown to enhance migration of human first trimester villous explants (Irving and Lala 1995) and human trophoblasts from the HTR8/SVneo cell line (McKinnon, Chakraborty et al. 2001), we hypothesised that IGF-II would increase EPC outgrowth *in vitro*. However, in the current study, IGF-II had no effect on murine EPC outgrowth nor on the differentiation of trophoblasts. In contrast, it was previously shown that IGF-II increased the number of TGCs per EPC and increased levels of ploidy, with no change in migration levels (Kanai-Azuma, Kanai et al. 1993). However, EPCs in the previous study were cultured in media containing FCS for 24 h to allow attachment, before being cultured in serum free media in the presence of IGF-II. This may alter the outcome of these experiments since culture on plastic rather than Matrigel or exposure to FCS can alter the response of trophoblasts in culture. It appears though, that IGF-II may act differently in mouse and human early trophoblasts *in vitro* or may require the presence of an additional unidentified factor to promote invasion.

This study is the first to detect *asHif-1α* in the murine placenta. It was previously reported that prolonged hypoxic exposure of lung alveolar epithelial cells increases *asHif-1α* (Uchida, Rossignol et al. 2004). It was suggested that asHIF-1α exposes AU rich elements in the 3'UTR of *Hif-1α* mRNA which decreases the stability of *Hif-1α* mRNA and finally decreases HIF-1α protein expression (Rossignol, Vache et al. 2002). Conversely, *Hif-2α* mRNA is not destabilised by asHIF-1α due to the lack of AU rich elements (Uchida, Rossignol et al. 2004). In fact *Hif-2α* transcription can increase under hypoxia (Uchida, Rossignol et al. 2004), perhaps due to HIF-2α increasing its own promoter activity (Sato, Tanaka et al. 2002). Since asHIF-1α contains a putative HRE it is assumed that both HIF-1 and HIF-2 are involved in the regulation of asHIF-1α expression thus creating a negative feedback loop of HIF regulation (Rossignol, Vache et al. 2002; Uchida, Rossignol et al. 2004). This is consistent with the response to prolonged exposure to low oxygen observed in the present study, in these conditions, *Hif-1α* levels decreased, whilst *Hif-2α* mRNA was unchanged. We suggest that in the cultured murine EPCs HIF-1α and/or HIF-2α act to increase *asHif-1α* which in turn decreases the stability of *Hif-1α* mRNA. Levels of HIF-1α and HIF-2α protein were not investigated in the current study due to the limited amount of protein for extraction from the EPCs. However, experiments utilising a murine trophoblast stem cell line would enable further study of the effects of prolonged hypoxia on the HIF system.

In the current study, *Hif-1α* mRNA and *Igf2* mRNA were reduced following prolonged culture in 1% oxygen, in the presence or absence of IGF-II. It is possible that *Igf2* expression in the EPC may be regulated by HIF-1, as has been reported in other cell types (Feldser, Agani et al. 1999). Further studies are required to confirm this. An

intriguing finding from this study is the interaction between HIF-2α and IGF-II, where, in 1% oxygen, IGF-II negatively regulated *Hif-2α* mRNA abundance. Previously, a variety of growth factors and hormones have been shown to upregulate HIF-α subunits in normoxic conditions. IGF-II, in particular, was previously shown to upregulate HIF-1α protein (Feldser, Agani et al. 1999). However, to date, there are no reports of negative regulation of HIF-α by members of the insulin-like growth factor family. This observed decrease in *Hif-2α* mRNA may be either from a decrease in transcription of *Hif-2α* or a decrease in its mRNA stability. Whichever is the case, we assume this will be associated with a decrease in HIF-2α protein and hence HIF-2α mediated transcription. Levels of *asHif-1α*, *Vegf* and *Slc2a1* mRNA were also lower in 1% oxygen cultures with addition of IGF-II. Whether this is due to a reduction in HIF-2 mediated gene expression in the presence of IGF-II remains to be determined.

Our data show that the early mouse post implantation site, prior to vascularisation of the yolk sac, is exposed to the low oxygen levels seen in the early human placenta. In addition, culturing murine EPCs in low oxygen conditions induces complex changes in components of the HIF system and HIF target genes, which appears to be associated with the induction of altered trophoblast differentiation. This study and its exploration of HIF regulation in the mouse placenta, has important implications in HIF research. It is particularly relevant in prolonged periods of hypoxia in development such as during the first 10 weeks of human pregnancy where the fetus and the placenta are experiencing a low oxygen environment, as well as in pregnancy disorders such as preeclampsia where placental ischaemia late in gestation is detrimental to pregnancy outcome.

Chapter 5
Discussion and Conclusion

5.1 Discussion

Blastocyst implantation and formation of the placenta are highly regulated processes. IGF-II, in particular, has been suggested as a central regulator of trophoblast function but IGF-II does not act alone. The studies outlined in this thesis describe the interactions between IGF-II and other important regulators including the uPA system, oxygen and HIFs. In particular, it has been demonstrated that a new media formulation consisting of IGF-II, in combination with uPA and plasminogen, improves blastocyst hatching and implantation. In addition, IGF-II and its receptors were co-localised to early mouse implantation sites, supporting their role in early placentation and suggest that IGF-II may also play a role in decidual angiogenesis. Finally we have demonstrated a novel interaction between IGF-II, oxygen and the HIF system in trophoblast migration, differentiation and gene regulation.

5.1.1 IGF-II, in combination with uPA and plasminogen, improves *in vitro* blastocyst development and subsequent implantation

Fifteen percent of all couples in Australia are infertile and turn to assisted reproductive technologies (ART) such as IVF. Currently 2-3% of all births each year in Australia are a result of ART and this number is increasing as women are deciding to have children later in life. However, IVF success rates are still low and there is a high risk of implantation failure. In those women who do become pregnant there is a high risk of pregnancy complications including preterm delivery, low birth weight, SGA and perinatal mortalilty (Doyle, Beral et al. 1992; Shevell, Malone et al. 2005; Poikkeus, Gissler et al. 2007; Reddy, Wapner et al. 2007). Optimisation of the *in vitro* embryo culture environment may act to improve embryo development and reverse some of these negative outcomes.

Previous studies have shown that a variety of growth factors and other molecules are important for blastocyst development and implantation including IGF-II (Harvey and Kaye 1992; Rappolee, Sturm et al. 1992; O'Neill 1997; Kurzawa, Glabowski et al. 2001; Pantaleon, Jericho et al. 2003), uPA (Kubo, Spindle et al. 1981; Axelrod 1985; Zhang, Kidder et al. 1994) and plasminogen (Menino and O'Claray 1986; Menino, Dyk et al. 1989). However, a combination of factors is likely required to reverse the detrimental effects of ART. In Chapter 2 we describe a new media formulation for *in vitro* embryo culture containing IGF-II, uPA and plasminogen. This new media formulation improves embryo development, blastocyst hatching and implantation rate to a greater degree than IGF-II alone, or uPA plus plasminogen without IGF-II.

Recently, a novel interaction between IGF2R and uPAR has been shown in rhabdomyosarcoma cells whereby IGF2R and uPAR interact on the cell surface to form part of a large plasma membrane complex (Nykjaer, Christensen et al. 1998; Godar, Horejsi et al. 1999; Gallicchio, Kaun et al. 2003; Kreiling, Byrd et al. 2003; Olson, Yammani et al. 2004). Upon binding their respective ligands, IGF-II and uPA, plasminogen can also bind and once activated, can go on to promote cellular migration. We hypothesise that this interaction also occurs on the trophectoderm of the blastocyst and would act to promote blastocyst hatching from the zona pellucida and assist in attachment and implantation into the uterus. *In vitro* cultured embryos are known to have reduced expression of endogenous IGF-II (Stojanov, Alechna et al. 1999) and uPA (Aflalo, Sod-Moriah et al. 2004; Aflalo, Sod-Moriah et al. 2005) and therefore the addition of these molecules to embryo culture media may be necessary to achieve successful implantation.

Indeed, this new media formulation, containing a combination of IGF-II, uPA and plasminogen, improved blastocyst hatching and increased subsequent pregnancy and implantation rates following embryo transfer. Future work is required to elucidate the mechanism by which these factors interact but it is possible that the formation of the IGF2R, uPAR complex is involved. Analysis of blastocyst development in the presence of antisense RNA, small interfering RNA or antibodies specific for IGF2R and/or uPAR would provide further evidence for the role of the IGF2R, uPAR complex. In addition, utilisation of Leu^{27} IGF-II, an analogue of IGF-II which is selective for IGF2R, but not IGF1R nor IR would complement the current findings.

Unfortunately, due to the strain of mice used, we were unable to assess the effects of this new embryo culture media on pregnancy success and long-term postnatal outcomes. Therefore this work will need to be performed in a different mouse strain in order to gain additional information. Nonetheless, treatment of embryos with IGF-II, uPA and plasminogen, in combination, nearly doubled the number of mice that were pregnant following embryo transfer and may therefore be useful in improving the high rate of implantation failure following ART.

Interestingly, although the combination media formulation significantly increased the number of implantations per embryos transferred, there was no difference in the number of pups per litter. This suggests that the maternal decidua may be responding differently to an embryo that has been exposed to IGF-II, uPA and plasminogen versus one that has not, and may be an area that requires further exploration.

5.1.2 Early mouse placentation and decidual vascular remodelling

In Chapter 3 an atlas of mouse placental development and decidual vascular remodelling from days 5.5 to 10.5 is provided. We found that, unlike in humans, mouse trophoblasts do not invade into the endometrium individually in early pregnancy but remain in close contact with the main giant cell layer. Later, peri/endovascular TGCs have been found ahead of the main giant cell layer on day 12.5 of gestation (Adamson, Lu et al. 2002). Therefore it appears that the initiation of TGC invasion away from the main TGC layer occurs between days 10.5 to 12.5.

In humans, trophoblasts invade into and remodel the maternal spiral arterioles early in pregnancy (Zhou, Genbacev et al. 1998). However, we show that the remodelling of the maternal vasculature in mice is initiated prior to endovascular invasion by the trophoblasts. The maternal vessels in the primary decidual zone (PDZ) appear to undergo angiogenesis and there is an increase in vessel number and diameter throughout early pregnancy. In addition, most of these vessels lack a smooth muscle layer throughout early pregnancy and hence can not be classified as spiral arterioles.

Despite the absence of TGCs invading into the vessels early in pregnancy, it is possible that they secrete a variety of factors such as proliferin, VEGF and IGF-II which may promote vascular remodelling in the decidua.

5.1.3 Role of IGF-II in early mouse placentation

Despite the evidence that IGF-II plays an important role in placentation, there have been very few studies examining its role in the early post-implantation period, when maximal trophoblast invasion and decidual remodelling occur. In Chapter 3 we

describe the immunohistochemical localisation of IGF-II and the type 1 and 2 IGF receptors in mouse implantation sites from days 5.5 to 10.5 of gestation.

IGF-II and the IGF1R were expressed in all three layers of the placenta throughout early murine pregnancy, whereas the expression of IGF2R was highest earlier in pregnancy and declined by day 10.5. This suggests that IGF-II may bind to either IGF1R or IGF2R to promote placentation. To date there have been no reports on the role of IGF1R mediated IGF-II actions in trophoblast cells, though it is likely to have proliferative effects. Binding of IGF-II to the IGF2R may result in clearance of IGF-II or promote trophoblast invasion via a signal transduction cascade, as seen previously in human EVTs (McKinnon, Chakraborty et al. 2001). Alternatively, IGF-II has been shown to promote the differentiation of trophoblasts into TGCs (Kanai-Azuma, Kanai et al. 1993). Therefore it is possible that IGF-II is able to induce trophoblast proliferation, invasion or differentiation depending on the receptors available, the trophoblast cell type and the interactions between IGF-II and other molecules.

IGF-II and its receptors were localised to the maternal decidua and, in particular to those cells immediately adjacent to the maternal blood vessels. IGF-II can increase angiogenesis in human hepatocellular carcinoma cells (Bae, Lee et al. 1998) and it is possible that it exerts effects in the maternal decidua. IGF-II may act via either of IGF1R, IGF2R or IR to upregulate VEGF, as shown previously in other cell types (Kwon, Kwon et al. 2004).

Since IGF-II and its receptors are localised to both the developing placenta and the maternal decidua it may therefore play roles in both placentation and angiogenesis in

the decidua. However, the mechanism of its actions and the receptor signalling pathways involved warrant further elucidation.

5.1.4 Oxygen and HIFs in the mouse placenta

During the first trimester in humans, trophoblasts invade into and plug the uterine spiral arterioles so the placenta initially develops in a low oxygen environment (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). However, in mice the oxygen tension has not yet been measured and the trophoblasts do not plug the spiral arterioles (Chapter 3). In Chapter 4 we utilised the hypoxia marker pimonidazole to demonstrate that the early mouse implantation site is exposed to oxygen tensions of less than 1.5% on days 6.5 – 7.5 of gestation, which is similar to that detected in the early human placenta (Rodesch, Simon et al. 1992; Jauniaux, Watson et al. 2000; Burton and Caniggia 2001). We hypothesise that in the mouse there is an oxygen gradient during these early stages of pregnancy and, as the decidual vessels undergo angiogenesis, the blood supply to the conceptus gradually increases.

The effects of hypoxia are mediated by HIFs. The experiments outlined in chapter 4 demonstrate that HIF-1α protein is localised to the EPC and the decidua in early murine implantation sites. In addition, HIF-1α was detected later in pregnancy when no hypoxic regions were detected. This suggests that HIF-1α may be regulated by milder hypoxia (>1.5% O_2) or that it is being induced in a non-hypoxic manner. Indeed IGF-II displayed a similar pattern of expression to HIF-1α and has previously been shown to be able to increase HIF-1α protein levels (Feldser, Agani et al. 1999).

However, it is likely that other non-hypoxic regulators such as hormones and other growth factors may also be involved.

5.1.5 IGF-II interacts with oxygen and HIFs in mouse trophoblast cells

To determine the effect of, and interaction between, oxygen and IGF-II, day 7.5 mouse EPCs were cultured in various oxygen concentrations either with or without the addition of IGF-II. We determined that culture in low oxygen (1%) for 3 days significantly reduced the area of EPC outgrowth and increased the expression of the spongiotrophoblast marker *Tpbp*, indicating that oxygen may affect differentiation of the trophoblasts. In addition this cell lineage would presumably be a non-invasive cell population and hence may account for the decrease in EPC outgrowth in 1% oxygen. Interestingly, IGF-II had no effect on EPC outgrowth or on the differentiation of trophoblasts. However, IGF-II may play a role in proliferation or may require the presence of an additional unidentified factor to exert its effects in mouse trophoblasts.

In chapter 4 we determined that prolonged exposure of trophoblasts to 1% oxygen significantly reduced their expression of *Hif-1*α, whilst levels of *Hif-2*α were unchanged. This may be a response to the prolonged exposure to low oxygen (3 days). It has been proposed that HIF-1α stabilisation is an acute response to hypoxia that diminishes under prolonged periods of low oxygen whereas HIF-2α levels can increase under milder hypoxia and continue to increase with time (reviewed in, (Lofstedt, Fredlund et al. 2007). This is due to a decrease in the stability of *Hif-1*α mRNA under prolonged periods of hypoxia. asHIF-1α is a recently discovered negative regulator of *Hif-1*α mRNA stability. It is upregulated by both HIF-1α and HIF-2α and can then go on to decrease *Hif-1*α mRNA stability by exposing AU rich

elements in its 3'UTR (Rossignol, Vache et al. 2002). Conversely, *Hif-2α* is unable to be destabilised as it lacks AU rich elements (Uchida, Rossignol et al. 2004). We suggest that this may be the mechanism by which *Hif-1α* mRNA levels are decreased in the EPC culture following prolonged exposure to 1% oxygen (Figure 5-1). However, further work is required to fully elucidate the effects of prolonged versus acute hypoxia on HIF mRNA and protein levels in trophoblasts and will require the use of a trophoblast cell line.

We also demonstrate that *Igf2* mRNA levels are also decreased following prolonged exposure to 1% oxygen. We hypothesise that *Igf2* mRNA expression is regulated by *Hif-1α,* as has been reported in other cell types (Feldser, Agani et al. 1999), such that as *Hif-1α* mRNA is destabilised, HIF-1α activity is reduced and thus there is a decrease in *Igf2* expression. However further studies are required to confirm this.

The most intriguing finding from Chapter 4 was that addition of exogenous IGF-II, only in 1% oxygen, significantly reduced the levels of *Hif-2α* mRNA. This is the first time that a growth factor has been demonstrated to negatively regulate the HIF system. Whether this is due to a decrease in *Hif-2α* transcription, or a decrease in its mRNA stability remains to be determined. We assume that this decrease in *Hif-2α* would also result in a decrease in HIF-2α mediated gene transcription. Indeed, levels of *asHif-1α*, *Vegf* and *Slc2a1* mRNA were also decreased in the 1% oxygen cultures with the addition of IGF-II (Figure 5-1).

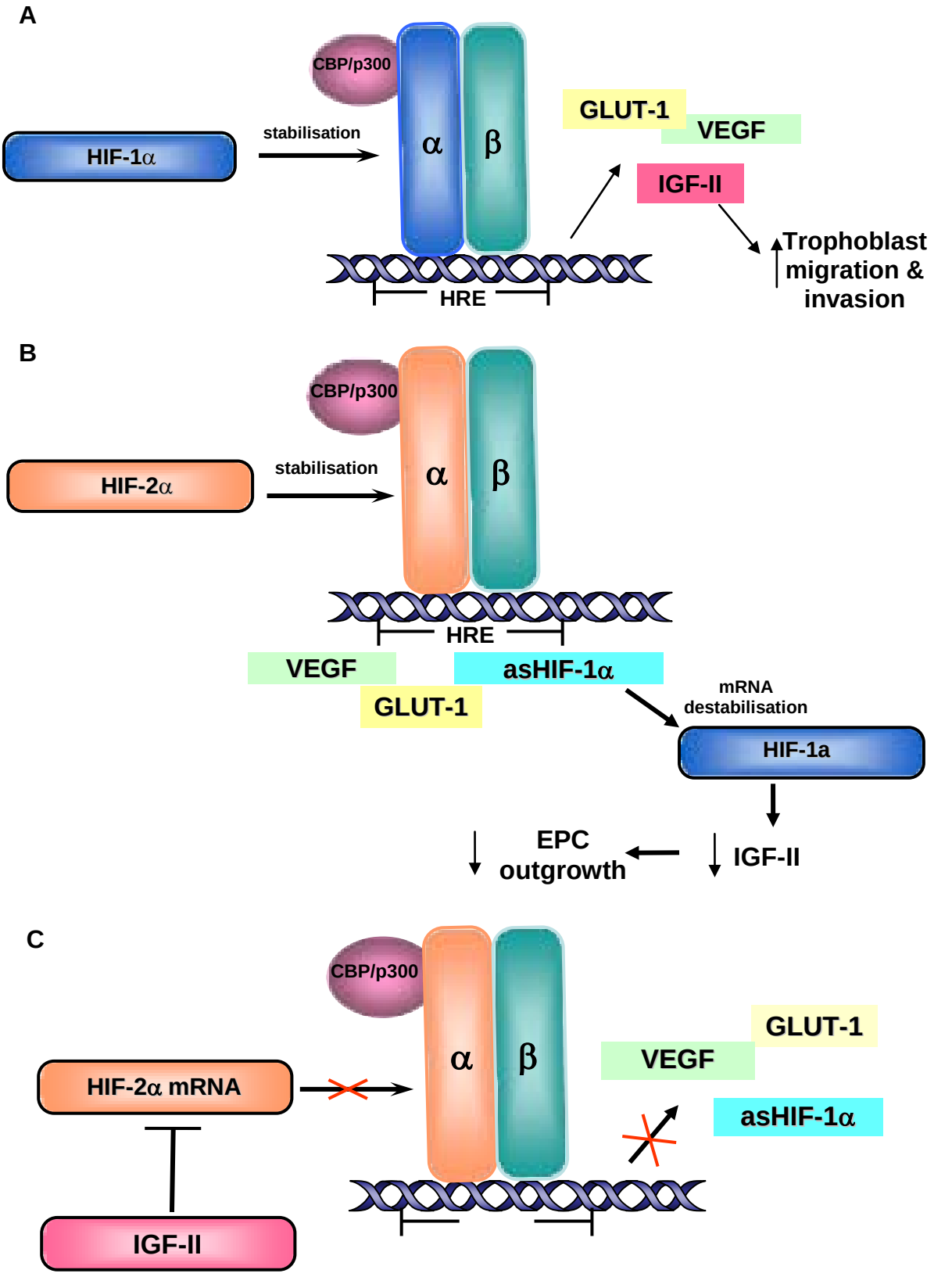


Figure 5-1 Proposed mechanism of HIF regulation under acute and prolonged hypoxia and with the addition of IGF-II

(**A**) Under acute hypoxia HIF-1α is stabilized and can induce the transcription of its target genes including *Vegf*, *Scl2a1* (GLUT-1) and Igf2. (**B**) Under prolonged hypoxia HIF-2α is stabilized and it can induce its target genes including *asHif-1α* which can then go on to destabilize *Hif-1α* mRNA. (**C**) Under prolonged low oxygen (1%) exogenous IGF-II negatively regulates *Hif-2α* mRNA which results in a decrease in HIF-2α mediated gene transcription.

These are very intriguing data but further work is required to elucidate the mechanism by which IGF-II and oxygen interact to negatively regulate the HIF-2 pathway. Subsequent studies in a trophoblast cell line examining the effects of oxygen and IGF-II in acute versus prolonged hypoxia are essential. This would allow us to more closely examine how IGF-II and oxygen interact to affect HIF mRNA and protein levels, as well as trophoblast behaviour *in vitro*. In particular, it would allow us to determine which receptor IGF-II is acting through and the mechanism by which it negatively regulates the HIF system. In addition, mice treated with exogenous IGF-II during pregnancy could be assessed to determine the effects of IGF-II on the HIF system and subsequent placental development *in vivo*.

5.2 IGF-II, a key regulator in blastocyst development, implantation and placentation in the mouse

The research outlined in this thesis has increased our knowledge and highlighted the importance of IGF-II in regulating blastocyst development, implantation and placentation. In addition, these studies suggest that the interactions of IGF-II with other molecules that regulate these processes are complex and require further investigation.

Poor placentation has been implicated in a variety of pregnancy complications including unexplained miscarriage, preeclampsia, IUGR and preterm birth. Interestingly, the risk of developing these pregnancy complications is increased following ART. The challenge then is to identify those women at risk of developing placental insufficiency and to improve ART to reduce these complications. We have determined that IGF-II, in combination with uPA and plasminogen, can improve implantation rates in mouse embryos. We therefore believe that this new media

formulation may one day be used in ART to reverse some of the detrimental effects of *in vitro* culture.

We also demonstrate that IGF-II and HIF-1α are abundantly expressed in the early mouse implantation site and that the early conceptus is exposed to a low oxygen environment. In the future it will be necessary to determine the exact oxygen concentration of the mouse implantation site, perhaps through the use of an oxygen probe, in order to validate our results and also to detect if there is an oxygen gradient in the implantation site. Nonetheless, our findings are similar to what is known about human placentation and therefore the mouse may be useful in examining the mechanisms by which IGF-II and oxygen regulate placental development. However, we have also shown several key differences between human and mouse placentation. Mouse trophoblasts do not invade the endometrium individually and, in addition, the maternal vasculature is remodelled independently of the trophoblasts early in pregnancy. Therefore, care needs to be taken when using mice as a model.

Importantly, this thesis describes novel interactions between IGFs and oxygen in their regulation of HIFs. This work has important implications for placental research, not only when considering the development of the placenta in the first trimester, but also when determining therapeutic interventions to prevent and/or treat pregnancy complications such preeclampsia. This disorder is characterised by prolonged hypoxia late in gestation due to insufficient remodelling of the spiral arterioles. Therefore, further research to the reveal molecular mechanisms by which oxygen and IGF-II interact during pregnancy are of critical importance. In addition, IGFBPs, the IGF receptors and regulators of HIF stabilisation and/or activity (for example,

VHL, PHDs, FIH, IPAS and asHIF-1α), all need to be taken into consideration as they may alter the response to IGF-II and/or oxygen (as discussed in Chapter 1).

Finally, non-hypoxic regulation of HIFs in the placenta also needs to be studied in more detail. As outlined in Chapter 1, HIFs can be upregulated by growth factors, cytokines or hormones and to date there have been very few studies investigating this mode of regulation in the placenta. In addition, it is clear from this study that differences between HIF-1α and -2α also require further investigation. HIF-1α and -2α can be regulated differently depending on the cell type, oxygen concentration and length of hypoxic exposure. We show here that they also respond differently to non-hypoxic stimuli. This work not only has implications in placental research, but also in cancer research as IGFs and HIFs and plasminogen activators are upregulated in many tumours.

The experiments described in this thesis have led us to reevaluate our perceptions of how oxygen regulates trophoblast behaviour. Currently, the majority of research in this area simply focuses on low oxygen and its role in trophoblast proliferation, invasion and differentiation. However, we need to refrain from categorizing placental events. For example, the first trimester is a very dynamic time period in placental growth and thus an event occurring in the 4th week of human gestation may not necessarily have the same effect as if it occurs in the 8th week. Therefore, caution needs to be taken in interpreting results when the concentration of oxygen, the length of hypoxic exposure and the presence of other cofactors may alter the experimental outcomes. This is particularly important given the complexity of the HIF system.

In addition, this thesis highlights the importance of studying the effects of, and interactions between, molecules rather than the effects of a single molecule. The regulation of implantation and placentation is a highly complex process and likely involves a combination of hormones, growth factors, cytokines and ECM molecules which interact with each other to exert their effects. It is of vital importance then to think more broadly about the molecules we study and the interactions they may have.

5.3 Conclusion

Treatment of embryos *in vitro* with a new media formulation containing IGF-II, uPA and plasminogen, in combination, improves blastocyst development, pregnancy and implantation rates following embryo transfer. We propose that this new media formulation could one day be used in ART to improve implantation rates and pregnancy outcome. The experiments in post-implantation mouse pregnancy have demonstrated that IGF-II, its receptors and HIF-1α are abundantly expressed in the implantation site and that the mouse conceptus develops in a low oxygen environment. In addition, we have demonstrated novel interactions between oxygen and IGF-II in HIF regulation in the mouse placenta.

Chapter 6
References

Adamson, S. L., Y. Lu, et al. (2002). "Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta." Dev Biol **250**(2): 358-73.

Adelman, D. M., M. Gertsenstein, et al. (2000). "Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses." Genes Dev **14**(24): 3191-203.

Aflalo, E. D., U. A. Sod-Moriah, et al. (2004). "Differences in the implantation rates of rat embryos developed in vivo and in vitro: possible role for plasminogen activators." Fertil Steril **81 Suppl 1**: 780-5.

Aflalo, E. D., U. A. Sod-Moriah, et al. (2005). "Expression of plasminogen activators in preimplantation rat embryos developed in vivo and in vitro." Reprod Biol Endocrinol **3**: 7.

Aflalo, E. D., U. A. Sod-Moriah, et al. (2007). "EGF increases expression and activity of PAs in preimplantation rat embryos and their implantation rate." Reprod Biol Endocrinol **5**: 4.

Akeno, N., J. Robins, et al. (2002). "Induction of vascular endothelial growth factor by IGF-I in osteoblast-like cells is mediated by the PI3K signaling pathway through the hypoxia-inducible factor-2alpha." Endocrinology **143**(2): 420-5.

Appelhoff, R. J., Y. M. Tian, et al. (2004). "Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor." J Biol Chem **279**(37): 38458-65.

Arany, Z., L. E. Huang, et al. (1996). "An essential role for p300/CBP in the cellular response to hypoxia." Proc Natl Acad Sci U S A **93**(23): 12969-73.

Axelrod, H. R. (1985). "Altered trophoblast functions in implantation-defective mouse embryos." Developmental Biology **108**(1): 185-190.

Bae, M. H., M. J. Lee, et al. (1998). "Insulin-like growth factor II (IGF-II) secreted from HepG2 human hepatocellular carcinoma cells shows angiogenic activity." Cancer Lett **128**(1): 41-6.

Baker, J., J. P. Liu, et al. (1993). "Role of insulin-like growth factors in embryonic and postnatal growth." Cell **75**(1): 73-82.

Balen, A. H., S. L. Tan, et al. (1993). "Miscarriage rates following in-vitro fertilization are increased in women with polycystic ovaries and reduced by pituitary desensitization with buserelin." Hum Reprod **8**(6): 959-64.

Bany, B. M., M. B. Harvey, et al. (2000). "Expression of matrix metalloproteinases 2 and 9 in the mouse uterus during implantation and oil-induced decidualization." J Reprod Fertil **120**(1): 125-34.

Bass, K. E., H. Li, et al. (1997). "Tissue inhibitor of metalloproteinase-3 expression is upregulated during human cytotrophoblast invasion in vitro." Dev Genet **21**(1): 61-7.

Bauer, S., J. Pollheimer, et al. (2004). "Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures." J Clin Endocrinol Metab **89**(2): 812-22.

Baxter, R. C. (2000). "Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities." Am J Physiol Endocrinol Metab **278**(6): E967-976.

Berra, E., E. Benizri, et al. (2003). "HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia." Embo J **22**(16): 4082-90.

Berra, E., D. Roux, et al. (2001). "Hypoxia-inducible factor-1 alpha (HIF-1 alpha) escapes O(2)-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm." EMBO Rep **2**(7): 615-20.

Bischof, P., M. Martelli, et al. (1995). "Importance of matrix metalloproteinases in human trophoblast invasion." Early Pregnancy **1**(4): 263-9.

Bischof, P., A. Meisser, et al. (1998). "Involvement of trophoblast in embryo implantation: regulation by paracrine factors." J Reprod Immunol **39**(1-2): 167-77.

Bischof, P., A. Meisser, et al. (2000). "Paracrine and autocrine regulators of trophoblast invasion - a review." Placenta **21**(Suppl A): S55-60.

Bjorn, S. F., N. Hastrup, et al. (2000). "Messenger RNA for membrane-type 2 matrix metalloproteinase, MT2-MMP, is expressed in human placenta of first trimester." Placenta **21**(2-3): 170-6.

Blankenship, T. N. and R. L. Given (1992). "Penetration of the uterine epithelial basement membrane during blastocyst implantation in the mouse." Anat Rec **233**(2): 196-204.

Bruick, R. K. and S. L. McKnight (2001). "A conserved family of prolyl-4-hydroxylases that modify HIF." Science **294**(5545): 1337-1340.

Bugge, T. H., M. J. Flick, et al. (1995). "Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction." Genes Dev **9**(7): 794-807.

Bugge, T. H., T. T. Suh, et al. (1995). "The Receptor for Urokinase-type Plasminogen Activator Is Not Essential for Mouse Development or Fertility." J. Biol. Chem. **270**(28): 16886-16894.

Burroughs, K. D., J. Oh, et al. (2003). "Phosphatidylinositol 3-kinase and mek1/2 are necessary for insulin-like growth factor-I-induced vascular endothelial growth factor synthesis in prostate epithelial cells: a role for hypoxia-inducible factor-1?" Mol Cancer Res **1**(4): 312-22.

Burton, G. J. and I. Caniggia (2001). "Hypoxia: implications for implantation to delivery-a workshop report." Placenta **22**(Suppl A): S63-5.

Burton, G. J., E. Jauniaux, et al. (1999). "Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited." Am J Obstet Gynecol **181**(3): 718-24.

Byrne, A. T., J. Southgate, et al. (2002). "Effects of insulin-like growth factors I and II on tumour-necrosis-factor-alpha-induced apoptosis in early murine embryos." Reprod Fertil Dev **14**(1-2): 79-83.

Campbell, P. G., J. F. Novak, et al. (1992). "Involvement of the plasmin system in dissociation of the insulin-like growth factor-binding protein complex." Endocrinology **130**(3): 1401-12.

Caniggia, I., H. Mostachfi, et al. (2000). "Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3)." J Clin Invest **105**(5): 577-87.

Caniggia, I. and J. L. Winter (2002). "Adriana and Luisa Castellucci Award lecture 2001. Hypoxia inducible factor-1: oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies - a review." Placenta **23**(Suppl A): S47-57.

Cannon, M. J. and A. R. Menino, Jr. (1998). "Changes in the bovine zona pellucida induced by plasmin or embryonic plasminogen activator." Mol Reprod Dev **51**(3): 330-8.

Carroll, V. A. and M. Ashcroft (2006). "Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway." Cancer Res **66**(12): 6264-70.

Carter, A. M., K. Nygard, et al. (2006). "The Expression of Insulin-like Growth Factor and Insulin-like Growth Factor Binding Protein mRNAs in Mouse Placenta." Placenta **27**(2-3): 278-290.

Chan, D. A., P. D. Sutphin, et al. (2005). "Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha." Mol. Cell. Biol. **25**(15): 6415-6426.

Chang, H., K. G. Shyu, et al. (2003). "Regulation of hypoxia-inducible factor-1 alpha by cyclical mechanical stretch in rat vascular smooth muscle cells." Clin Sci (Lond) **105**(4): 447-56.

Chavez, J. C. and J. C. LaManna (2002). "Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: potential role of insulin-like growth factor-1." J Neurosci **22**(20): 8922-31.

Chi, M. M.-Y., A. L. Schlein, et al. (2000). "High Insulin-Like Growth Factor 1 (IGF-1) and Insulin Concentrations Trigger Apoptosis in the Mouse Blastocyst via Down-Regulation of the IGF-1 Receptor." Endocrinology **141**(12): 4784-4792.

Chilov, D., G. Camenisch, et al. (1999). "Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha." J Cell Sci **112 (Pt 8)**: 1203-12.

Clemmons, D. R. (1998). "Role of insulin-like growth factor binding proteins in controlling IGF actions." Mol Cell Endocrinol **140**(1-2): 19-24.

Coan, P. M., N. Conroy, et al. (2006). "Origin and characteristics of glycogen cells in the developing murine placenta." Dev Dyn **235**(12): 3280-94.

Cockman, M. E., N. Masson, et al. (2000). "Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein." J Biol Chem **275**(33): 25733-41.

Compernolle, V., K. Brusselmans, et al. (2002). "Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice." Nat Med **8**(7): 702-10.

Constancia, M., M. Hemberger, et al. (2002). "Placental-specific IGF-II is a major modulator of placental and fetal growth." Nature **417**(6892): 945-8.

Coppens, M., P. Loquet, et al. (1996). "Longitudinal evaluation of uteroplacental and umbilical blood flow changes in normal early pregnancy." Ultrasound in Obstetrics and Gynecology **7**(2): 114-121.

Cowden Dahl, K. D., B. H. Fryer, et al. (2005). "Hypoxia-Inducible Factors 1 alpha and 2 alpha Regulate Trophoblast Differentiation." Mol. Cell. Biol. **25**(23): 10479-10491.

Crocker, I. P., M. Wareing, et al. (2005). "The effect of vascular origin, oxygen, and tumour necrosis factor alpha on trophoblast invasion of maternal arteries in vitro." J Pathol **206**(4): 476-85.

Cross, J. C., M. Hemberger, et al. (2002). "Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta." Mol Cell Endocrinol **187**(1-2): 207-12.

Cross, J. C., Z. Werb, et al. (1994). "Implantation and the placenta: key pieces of the development puzzle." Science **266**(5190): 1508-18.

Crossey, P. A., C. C. Pillai, et al. (2002). "Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice." J Clin Invest **110**(3): 411-8.

D'Ercole, A. J. (1996). Insulin-like growth factors and their receptors in growth. Endocrinology and Metabolism Clinics of North America. J. Vassalo. Philadelphia, WB Saunders Company. **25;3:** 573-590.

Daikoku, T., H. Matsumoto, et al. (2003). "Expression of hypoxia-inducible factors in the peri-implantation mouse uterus is regulated in a cell-specific and ovarian steroid hormone-dependent manner. Evidence for differential function of HIFs during early pregnancy." J Biol Chem **278**(9): 7683-91.

Dames, S. A., M. Martinez-Yamout, et al. (2002). "Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response." Proc Natl Acad Sci U S A **99**(8): 5271-6.

Damsky, C. H., M. L. Fitzgerald, et al. (1992). "Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo." J Clin Invest **89**(1): 210-22.

Das, S. K., S. Yano, et al. (1997). "Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period." Dev Genet **21**(1): 44-54.

DeChiara, T. M., A. Efstratiadis, et al. (1990). "A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting." Nature **345**(6270): 78-80.

del Peso, L., M. C. Castellanos, et al. (2003). "The von Hippel Lindau/Hypoxia-inducible Factor (HIF) Pathway Regulates the Transcription of the HIF-Proline Hydroxylase Genes in Response to Low Oxygen." J. Biol. Chem. **278**(49): 48690-48695.

Denley, A., L. J. Cosgrove, et al. (2005). "Molecular interactions of the IGF system." Cytokine & Growth Factor Reviews **16**(4-5): 421-439.

Dommisse, J. and A. J. Tiltman (1992). "Placental bed biopsies in placental abruption." Br J Obstet Gynaecol **99**(8): 651-4.

Doyle, P., V. Beral, et al. (1992). "Preterm delivery, low birthweight and small-for-gestational-age in liveborn singleton babies resulting from in-vitro fertilization." Hum. Reprod. **7**(3): 425-428.

Duan, C. and Q. Xu (2005). "Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions." Gen Comp Endocrinol **142**(1-2): 44-52.

Ema, M., K. Hirota, et al. (1999). "Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300." Embo J **18**(7): 1905-14.

Ema, M., S. Taya, et al. (1997). "A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development." Proc Natl Acad Sci U S A **94**(9): 4273-8.

Enders, A. C. and T. N. Blankenship (1999). "Comparative placental structure." Adv Drug Deliv Rev **38**(1): 3-15.

Eng, G. S., R. A. Sheridan, et al. (2007). "AMP kinase activation increases glucose uptake, decreases apoptosis, and improves pregnancy outcome in embryos exposed to high IGF-I concentrations." Diabetes **56**(9): 2228-34.

Epstein, A. C. R., J. M. Gleadle, et al. (2001). "C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation." Cell **107**(1): 43-54.

Fabian, D., G. Il'kova, et al. (2004). "Inhibitory effect of IGF-I on induced apoptosis in mouse preimplantation embryos cultured in vitro." Theriogenology **61**(4): 745-55.

Feinberg, R. F., L. C. Kao, et al. (1989). "Plasminogen activator inhibitor types 1 and 2 in human trophoblasts. PAI-1 is an immunocytochemical marker of invading trophoblasts." Lab Invest **61**(1): 20-6.

Feldser, D., F. Agani, et al. (1999). "Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2." Cancer Res **59**(16): 3915-8.

Fisher, S. J., T. Y. Cui, et al. (1989). "Adhesive and degradative properties of human placental cytotrophoblast cells in vitro." J Cell Biol **109**(2): 891-902.

Fitzpatrick, T. E. and C. H. Graham (1998). "Stimulation of plasminogen activator inhibitor-1 expression in immortalized human trophoblast cells cultured under low levels of oxygen." Exp Cell Res **245**(1): 155-62.

Flamme, I., T. Frohlich, et al. (1997). "HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1[alpha] and developmentally expressed in blood vessels." Mechanisms of Development **63**(1): 51-60.

Floridon, C., O. Nielsen, et al. (1999). "Localization and significance of urokinase plasminogen activator and its receptor in placental tissue from intrauterine, ectopic and molar pregnancies." Placenta **20**(8): 711-21.

Floridon, C., O. Nielsen, et al. (2000). "Does plasminogen activator inhibitor-1 (PAI-1) control trophoblast invasion? A study of fetal and maternal tissue in intrauterine, tubal and molar pregnancies." Placenta **21**(8): 754-62.

Foidart, J. M., J. Hustin, et al. (1992). "The human placenta becomes haemochorial at the 13th week of pregnancy." Int J Dev Biol **36**(3): 451-3.

Freedman, S. J., Z. Y. Sun, et al. (2002). "Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha." Proc Natl Acad Sci U S A **99**(8): 5367-72.

Freeman, H., K. Shimomura, et al. (2006). "Nicotinamide nucleotide transhydrogenase: a link between insulin secretion, glucose metabolism and oxidative stress." Biochem Soc Trans **34**(Pt 5): 806-10.

Freeman, H. C., A. Hugill, et al. (2006). "Deletion of nicotinamide nucleotide transhydrogenase: a new quantitive trait locus accounting for glucose intolerance in C57BL/6J mice." Diabetes **55**(7): 2153-6.

Fukuda, R., K. Hirota, et al. (2002). "Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells." J Biol Chem **277**(41): 38205-11.

Fukuda, R., B. Kelly, et al. (2003). "Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1." Cancer Res **63**(9): 2330-4.

Gallicchio, M. A., C. Kaun, et al. (2003). "Urokinase type plasminogen activator receptor is involved in insulin-like growth factor-induced migration of rhabdomyosarcoma cells in vitro." J Cell Physiol **197**(1): 131-8.

Gargosky, S. E., K. J. Moyse, et al. (1990). "Circulating levels of insulin-like growth factors increase and molecular forms of their serum binding proteins change with human pregnancy." Biochemical and Biophysical Research Communications **170**(3): 1157-1163.

Genbacev, O., R. Joslin, et al. (1996). "Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia." J Clin Invest **97**(2): 540-50.

Genbacev, O., A. Krtolica, et al. (2001). "Human cytotrophoblast expression of the von Hippel-Lindau protein is downregulated during uterine invasion in situ and upregulated by hypoxia in vitro." Dev Biol **233**(2): 526-36.

Genbacev, O., Y. Zhou, et al. (1997). "Regulation of human placental development by oxygen tension." Science **277**(5332): 1669-72.

Georgiades, P., A. C. Ferguson-Smith, et al. (2002). "Comparative developmental anatomy of the murine and human definitive placentae." Placenta **23**(1): 3-19.

Germain-Lee, E. L., M. Janicot, et al. (1992). "Expression of a type I insulin-like growth factor receptor with low affinity for insulin-like growth factor II." Biochem J **281 (Pt 2)**: 413-7.

Gibson, J. M., J. D. Aplin, et al. (2001). "Regulation of IGF bioavailability in pregnancy." Mol Hum Reprod **7**(1): 79-87.

Gicquel, C. and Y. Le Bouc (2006). "Hormonal Regulation of Fetal Growth." Hormone Research **65**: 28-33.

Glabowski, W., R. Kurzawa, et al. (2005). "Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha." Reprod Biol **5**(1): 83-99.

Gnarra, J. R., J. M. Ward, et al. (1997). "Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice." Proc Natl Acad Sci U S A **94**(17): 9102-7.

Godar, S., V. Horejsi, et al. (1999). "M6P/IGFII-receptor complexes urokinase receptor and plasminogen for activation of transforming growth factor-beta1." Eur J Immunol **29**(3): 1004-13.

Gorlach, A., I. Diebold, et al. (2001). "Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase." Circ Res **89**(1): 47-54.

Gothie, E., D. E. Richard, et al. (2000). "Identification of alternative spliced variants of human hypoxia-inducible factor-1alpha." J Biol Chem **275**(10): 6922-7.

Graham, C. H., I. Connelly, et al. (1994). "Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta." Exp Cell Res **214**(1): 93-9.

Graham, C. H., T. E. Fitzpatrick, et al. (1998). "Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway." Blood **91**(9): 3300-7.

Graham, C. H., L. M. Postovit, et al. (2000). "Adriana and Luisa Castellucci award lecture 1999: role of oxygen in the regulation of trophoblast gene expression and invasion." Placenta **21**(5-6): 443-50.

Gratton, R. J., H. Asano, et al. (2002). "The regional expression of insulin-like growth factor II (IGF-II) and insulin-like growth factor binding protein-1 (IGFBP-1) in the placentae of women with pre-eclampsia." Placenta **23**(4): 303-10.

Groskopf, J. C., L. J. Syu, et al. (1997). "Proliferin induces endothelial cell chemotaxis through a G protein-coupled, mitogen-activated protein kinase-dependent pathway." Endocrinology **138**(7): 2835-40.

Groulx, I. and S. Lee (2002). "Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor requires nuclear-cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein." Mol Cell Biol **22**(15): 5319-36.

Gu, Y. Z., S. M. Moran, et al. (1998). "Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha." Gene Expr **7**(3): 205-13.

Gultice, A. D., K. L. Selesniemi, et al. (2006). "Hypoxia Inhibits Differentiation of Lineage-Specific Rcho-1 Trophoblast Giant Cells." Biol Reprod **74**(6): 1041-1050.

Hamilton, G. S., J. J. Lysiak, et al. (1998). "Autocrine-paracrine regulation of human trophoblast invasiveness by insulin-like growth factor (IGF)-II and IGF-binding protein (IGFBP)-1." Exp Cell Res **244**(1): 147-56.

Han, V. K., N. Bassett, et al. (1996). "The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface." J Clin Endocrinol Metab **81**(7): 2680-93.

Han, V. K. and A. M. Carter (2000). "Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals." Placenta **21**(4): 289-305.

Hara, S., J. Hamada, et al. (2001). "Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha." Biochem Biophys Res Commun **287**(4): 808-13.

Harvey, M. B. and P. L. Kaye (1991). "IGF-2 receptors are first expressed at the 2-cell stage of mouse development." Development **111**(4): 1057-60.

Harvey, M. B. and P. L. Kaye (1992). "IGF-2 stimulates growth and metabolism of early mouse embryos." Mech Dev **38**(3): 169-73.

Harvey, M. B. and P. L. Kaye (1992). "Mediation of the actions of insulin and insulin-like growth factor-1 on preimplantation mouse embryos in vitro." Mol Reprod Dev **33**(3): 270-5.

Harvey, M. B., K. J. Leco, et al. (1995). "Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor." Development **121**(4): 1005-14.

Hayashi, M., M. Sakata, et al. (2005). "Hypoxia Up-Regulates Hypoxia-Inducible Factor-1{alpha} Expression through RhoA Activation in Trophoblast Cells." J Clin Endocrinol Metab **90**(3): 1712-1719.

Hayashi, M., M. Sakata, et al. (2004). "Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells." J Endocrinol **183**(1): 145-54.

Hayati, A. R., F. C. Cheah, et al. (2007). "Insulin-like growth factor-1 receptor expression in the placentae of diabetic and normal pregnancies." Early Hum Dev **83**(1): 41-6.

Heidbreder, M., F. Frohlich, et al. (2003). "Hypoxia rapidly activates HIF-3α mRNA expression." FASEB J.: 02-0963fje.

Hellwig-Burgel, T., K. Rutkowski, et al. (1999). "Interleukin-1beta and Tumor Necrosis Factor-alpha Stimulate DNA Binding of Hypoxia-Inducible Factor-1." Blood **94**(5): 1561-1567.

Herr, F., O. D. Liang, et al. (2003). "Possible angiogenic roles of insulin-like growth factor II and its receptors in uterine vascular adaptation to pregnancy." J Clin Endocrinol Metab **88**(10): 4811-7.

Hewitson, K. S., L. A. McNeill, et al. (2002). "Hypoxia-inducible Factor (HIF) Asparagine Hydroxylase Is Identical to Factor Inhibiting HIF (FIH) and Is Related to the Cupin Structural Family." J. Biol. Chem. **277**(29): 26351-26355.

Hills, F. A., M. G. Elder, et al. (2004). "Regulation of human villous trophoblast by insulin-like growth factors and insulin-like growth factor-binding protein-1." J Endocrinol **183**(3): 487-96.

Hills, F. A., J. English, et al. (1996). "Circulating levels of IGF-I and IGF-binding protein-1 throughout pregnancy: relation to birthweight and maternal weight." J Endocrinol **148**(2): 303-309.

Holmquist-Mengelbier, L., E. Fredlund, et al. (2006). "Recruitment of HIF-1[alpha] and HIF-2[alpha] to common target genes is differentially regulated in neuroblastoma: HIF-2[alpha] promotes an aggressive phenotype." Cancer Cell **10**(5): 413-423.

Huang, J., Q. Zhao, et al. (2002). "Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3." J Biol Chem **277**(42): 39792-800.

Huang, L. E. and H. F. Bunn (2003). "Hypoxia-inducible factor and its biomedical relevance." J Biol Chem **14**: 14.

Huang, L. E., J. Gu, et al. (1998). "Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway." PNAS **95**(14): 7987-7992.

Huppertz, B., S. Kertschanska, et al. (1998). "Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta." Cell Tissue Res **291**(1): 133-48.

Hustin, J. and J. P. Schaaps (1987). "Echographic [corrected] and anatomic studies of the maternotrophoblastic border during the first trimester of pregnancy." Am J Obstet Gynecol **157**(1): 162-8.

Ikezu, T., T. Okamoto, et al. (1995). "In Vivo Coupling of Insulin-like Growth Factor II/Mannose 6-Phosphate Receptor to Heteromeric G Proteins." J. Biol. Chem. **270**(49): 29224-29228.

Irving, J. A. and P. K. Lala (1995). "Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1." Exp Cell Res **217**(2): 419-27.

Irwin, J. C., L. F. Suen, et al. (2001). "Insulin-like growth factor (IGF)-II inhibition of endometrial stromal cell tissue inhibitor of metalloproteinase-3 and IGF-binding protein-1 suggests paracrine interactions at the decidua:trophoblast interface during human implantation." J Clin Endocrinol Metab **86**(5): 2060-4.

Isaka, K., S. Usuda, et al. (2003). "Expression and activity of matrix metalloproteinase 2 and 9 in human trophoblasts." Placenta **24**(1): 53-64.

Ivan, M., K. Kondo, et al. (2001). "HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing." Science **292**(5516): 464-8.

Iyer, N. V., L. E. Kotch, et al. (1998). "Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha." Genes Dev **12**(2): 149-62.

Iyer, N. V., S. W. Leung, et al. (1998). "The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation." Genomics **52**(2): 159-65.

Jaakkola, P., D. R. Mole, et al. (2001). "Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation." Science **292**(5516): 468-72.

Jackson, D., O. V. Volpert, et al. (1994). "Stimulation and inhibition of angiogenesis by placental proliferin and proliferin-related protein." Science **266**(5190): 1581-4.

Jain, S., E. Maltepe, et al. (1998). "Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse." Mech Dev **73**(1): 117-23.

James, J. L., P. R. Stone, et al. (2006). "The effects of oxygen concentration and gestational age on extravillous trophoblast outgrowth in a human first trimester villous explant model." Hum. Reprod. **21**(10): 2699-2705.

Jauniaux, E., J. Hempstock, et al. (2003). "Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies." Am J Pathol **162**(1): 115-25.

Jauniaux, E., A. L. Watson, et al. (2000). "Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure." Am J Pathol **157**(6): 2111-22.

Jiang, B.-H., J. Z. Zheng, et al. (1997). "Transactivation and Inhibitory Domains of Hypoxia-Inducible Factor 1alpha . Modulation of Transcriptional Activity by Oxygen Tension." J. Biol. Chem. **272**(31): 19253-19260.

Jiang, B. H., G. Jiang, et al. (2001). "Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1." Cell Growth Differ **12**(7): 363-9.

Jones, J. I. and D. R. Clemmons (1995). "Insulin-like growth factors and their binding proteins: biological actions." Endocr Rev **16**(1): 3-34.

Kallio, P. J., I. Pongratz, et al. (1997). "Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor." Proc Natl Acad Sci U S A **94**(11): 5667-72.

Kallio, P. J., W. J. Wilson, et al. (1999). "Regulation of the Hypoxia-inducible Transcription Factor 1alpha by the Ubiquitin-Proteasome Pathway." J. Biol. Chem. **274**(10): 6519-6525.

Kanai-Azuma, M., Y. Kanai, et al. (1993). "Insulin-like growth factor (IGF)-I stimulates proliferation and migration of mouse ectoplacental cone cells, while IGF-II transforms them into trophoblastic giant cells in vitro." Biol Reprod **48**(2): 252-61.

Kawano, Y., S. Nakamura, et al. (2004). "The effect of insulin-like growth factor-I on production of vascular endothelial growth factor by amnion-derived (WISH) cells." Growth Hormone & IGF Research **14**(5): 376-381.

Khamsi, F., D. T. Armstrong, et al. (1996). "Expression of urokinase-type plasminogen activator in human preimplantation embryos." Mol Hum Reprod **2**(4): 273-6.

Khong, T. Y., F. De Wolf, et al. (1986). "Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants." Br J Obstet Gynaecol **93**(10): 1049-59.

Khong, T. Y., H. S. Liddell, et al. (1987). "Defective haemochorial placentation as a cause of miscarriage: a preliminary study." Br J Obstet Gynaecol **94**(7): 649-55.

Kiess, W., G. D. Blickenstaff, et al. (1988). "Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor." J Biol Chem **263**(19): 9339-44.

Kilburn, B. A., J. Wang, et al. (2000). "Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line." Biol Reprod **62**(3): 739-47.

Kim, H. J. and T. Y. Kim (2005). "Regulation of vascular endothelial growth factor expression by insulin-like growth factor-II in human keratinocytes, differential involvement of mitogen-activated protein kinases and feedback inhibition of protein kinase C." Br J Dermatol **152**(3): 418-25.

Kim, Y. M., E. Bujold, et al. (2003). "Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes." Am J Obstet Gynecol **189**(4): 1063-9.

Kim, Y. M., T. Chaiworapongsa, et al. (2002). "Failure of physiologic transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes." Am J Obstet Gynecol **187**(5): 1137-42.

Kjoller, L., S. M. Kanse, et al. (1997). "Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation." Exp Cell Res **232**(2): 420-9.

Koklanaris, N., J. C. Nwachukwu, et al. (2006). "First-trimester trophoblast cell model gene response to hypoxia." American Journal of Obstetrics and Gynecology **194**(3): 687-693.

Kozak, K. R., B. Abbott, et al. (1997). "ARNT-deficient mice and placental differentiation." Dev Biol **191**(2): 297-305.

Kreiling, J. L., J. C. Byrd, et al. (2003). "Binding of urokinase-type plasminogen activator receptor (uPAR) to the mannose 6-phosphate/insulin-like growth factor II receptor: contrasting interactions of full-length and soluble forms of uPAR." J Biol Chem **278**(23): 20628-37.

Kubo, H., A. Spindle, et al. (1981). "Inhibition of mouse blastocyst attachment and outgrowth by protease inhibitors." J Exp Zool **216**(3): 445-51.

Kurzawa, R., W. Glabowski, et al. (2001). "Evaluation of mouse preimplantation embryos cultured in media enriched with insulin-like growth factors I and II, epidermal growth factor and tumor necrosis factor alpha." Folia Histochem Cytobiol **39**(3): 245-51.

Kwon, Y. W., K. S. Kwon, et al. (2004). "Insulin-like growth factor-II regulates the expression of vascular endothelial growth factor by the human keratinocyte cell line HaCaT." J Invest Dermatol **123**(1): 152-8.

Lacey, H., T. Haigh, et al. (2002). "Mesenchymally-derived Insulin-like growth factor 1 provides a paracrine stimulus for trophoblast migration." BMC Dev Biol **2**(1): 5.

Lala, P. K. and C. Chakraborty (2003). "Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury." Placenta **24**(6): 575-87.

Lala, P. K. and C. H. Graham (1990). "Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors." Cancer Metastasis Rev **9**(4): 369-79.

Lando, D., D. J. Peet, et al. (2002). "FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor." Genes Dev **16**(12): 1466-71.

Lando, D., D. J. Peet, et al. (2002). "Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch." Science **295**(5556): 858-61.

Lau, M. M., C. E. Stewart, et al. (1994). "Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality." Genes Dev **8**(24): 2953-63.

Lee, S. J., F. Talamantes, et al. (1988). "Trophoblastic giant cells of the mouse placenta as the site of proliferin synthesis." Endocrinology **122**(5): 1761-8.

Li, Z., D. Wang, et al. (2005). "VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha." EMBO Rep **6**(4): 373-8.

Librach, C. L., Z. Werb, et al. (1991). "92-kD type IV collagenase mediates invasion of human cytotrophoblasts." J Cell Biol **113**(2): 437-49.

Lin, S., T. C. Thomas, et al. (2000). "Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice." Int J Obes Relat Metab Disord **24**(5): 639-46.

Lin, T. C., J. M. Yen, et al. (2003). "IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line." BMC Cell Biol **4**(1): 14.

Linzer, D. I., S. J. Lee, et al. (1985). "Identification of proliferin mRNA and protein in mouse placenta." Proc Natl Acad Sci U S A **82**(13): 4356-9.

Lisztwan, J., G. Imbert, et al. (1999). "The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity." Genes Dev. **13**(14): 1822-1833.

Liu, J., C. Chakraborty, et al. (2003). "Noncatalytic domain of uPA stimulates human extravillous trophoblast migration by using phospholipase C, phosphatidylinositol 3-kinase and mitogen-activated protein kinase." Exp Cell Res **286**(1): 138-51.

Liu, J. P., J. Baker, et al. (1993). "Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r)." Cell **75**(1): 59-72.

Lofstedt, T., E. Fredlund, et al. (2007). "Hypoxia inducible factor-2alpha in cancer." Cell Cycle **6**(8): 919-26.

Lopez, M. F., P. Dikkes, et al. (1996). "Insulin-like growth factor II affects the appearance and glycogen content of glycogen cells in the murine placenta." Endocrinology **137**(5): 2100-8.

Louvi, A., D. Accili, et al. (1997). "Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development." Dev Biol **189**(1): 33-48.

Ludwig, T., J. Eggenschwiler, et al. (1996). "Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds." Dev Biol **177**(2): 517-35.

Ludwig, T., R. Le Borgne, et al. (1995). "Roles for mannose-6-phosphate receptors in lysosomal enzyme sorting, IGF-II binding and clathrin-coat assembly." Trends Cell Biol **5**(5): 202-6.

Mahon, P. C., K. Hirota, et al. (2001). "FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity." Genes Dev **15**(20): 2675-86.

Makino, Y., R. Cao, et al. (2001). "Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression." Nature **414**(6863): 550-554.

Makino, Y., A. Kanopka, et al. (2002). "Inhibitory PAS Domain Protein (IPAS) Is a Hypoxia-inducible Splicing Variant of the Hypoxia-inducible Factor-3alpha Locus." J. Biol. Chem. **277**(36): 32405-32408.

Maltepe, E., G. W. Krampitz, et al. (2005). "Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta." Development **132**(15): 3393-3403.

Markham, K. and P. Kaye (2003). "Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst." Reproduction **125**(3): 327-336.

Martin, D. C., J. L. Fowlkes, et al. (1999). "Insulin-like growth factor II signaling in neoplastic proliferation is blocked by transgenic expression of the metalloproteinase inhibitor TIMP-1." J Cell Biol **146**(4): 881-92.

Marxsen, J. H., P. Stengel, et al. (2004). "Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases." Biochem J **381**(Pt 3): 761-7.

Masson, N., C. Willam, et al. (2001). "Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation." Embo J **20**(18): 5197-206.

Maxwell, P. H., M. S. Wiesener, et al. (1999). "The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis." Nature **399**(6733): 271-5.

McKinnon, T., C. Chakraborty, et al. (2001). "Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK." J Clin Endocrinol Metab **86**(8): 3665-74.

Meade, E. S., Y. Y. Ma, et al. (2007). "Role of hypoxia-inducible transcription factors 1alpha and 2alpha in the regulation of plasminogen activator inhibitor-1 expression in a human trophoblast cell line." Placenta **28**(10): 1012-9.

Mekhail, K., L. Gunaratnam, et al. (2004). "HIF activation by pH-dependent nucleolar sequestration of VHL." Nat Cell Biol **6**(7): 642-7.

Menino, A. R., Jr., A. R. Dyk, et al. (1989). "The effects of plasminogen on in vitro ovine embryo development." Biol Reprod **41**(5): 899-905.

Menino, A. R., Jr. and J. L. O'Claray (1986). "Enhancement of hatching and trophoblastic outgrowth by mouse embryos cultured in Whitten's medium containing plasmin and plasminogen." J Reprod Fertil **77**(1): 159-67.

Metzen, E., U. Berchner-Pfannschmidt, et al. (2003). "Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing." J Cell Sci **116**(Pt 7): 1319-26.

Minet, E., G. Michel, et al. (2000). "Role of HIF-1 as a transcription factor involved in embryonic development, cancer progression and apoptosis (review)." Int J Mol Med **5**(3): 253-9.

Mohan, S. and D. J. Baylink (2002). "IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms." J Endocrinol **175**(1): 19-31.

Mole, D. R., C. W. Pugh, et al. (2002). "Regulation of the HIF pathway: enzymatic hydroxylation of a conserved prolyl residue in hypoxia-inducible factor alpha subunits governs capture by the pVHL E3 ubiquitin ligase complex." Adv Enzyme Regul **42**: 333-47.

Moore, K. (1974). The Developing Human, W.B. Saunders Company.

Moore, K. (1988). The Developing Human, W.B. Saunders Company.

Morgan, D. O., J. C. Edman, et al. (1987). "Insulin-like growth factor II receptor as a multifunctional binding protein." Nature **329**(6137): 301-7.

Morgan, D. O., K. Jarnagin, et al. (1986). "Purification and characterization of the receptor for insulin-like growth factor I." Biochemistry **25**(19): 5560-4.

Morrish, D. W., J. Dakour, et al. (1998). "Functional regulation of human trophoblast differentiation." J Reprod Immunol **39**(1-2): 179-95.

Multhaupt, H. A., A. Mazar, et al. (1994). "Expression of urokinase receptors by human trophoblast. A histochemical and ultrastructural analysis." Lab Invest **71**(3): 392-400.

Muntener, M. and Y. C. Hsu (1977). "Development of trophoblast and placenta of the mouse. A reinvestigation with regard to the in vitro culture of mouse trophoblast and placenta." Acta Anat (Basel) **98**(3): 241-52.

Murayama, Y., T. Okamoto, et al. (1990). "Distinctive regulation of the functional linkage between the human cation-independent mannose 6-phosphate receptor and GTP-binding proteins by insulin-like growth factor II and mannose 6-phosphate." J. Biol. Chem. **265**(29): 17456-17462.

Nagy, A., Gertsenstein, M., Vintersten, K., Behringer, R. (2003). Manipulating the mouse embryo: a laboratory manual, Cold Spring Harbor Laboratory Press.

Nakayama, K., I. J. Frew, et al. (2004). "Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia." Cell **117**(7): 941-52.

Nissley, S. (1999). Type 2 IGF Receptor-Mediated Events. Contemporary Endocrinology: The IGF System. R. G. Rosenfeld, Roberts Jr., C.T., Humana Press Inc.

Nykjaer, A., E. I. Christensen, et al. (1998). "Mannose 6-phosphate/insulin-like growth factor-II receptor targets the urokinase receptor to lysosomes via a novel binding interaction." J Cell Biol **141**(3): 815-28.

O'Dell, S. D. and I. N. Day (1998). "Insulin-like growth factor II (IGF-II)." Int J Biochem Cell Biol **30**(7): 767-71.

O'Neill, C. (1997). "Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro." Biol Reprod **56**(1): 229-237.

O'Rourke, J. F., Y.-M. Tian, et al. (1999). "Oxygen-regulated and Transactivating Domains in Endothelial PAS Protein 1: Comparison with Hypoxia-inducible Factor-1alpha." J. Biol. Chem. **274**(4): 2060-2071.

Oehme, F., P. Ellinghaus, et al. (2002). "Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing." Biochem Biophys Res Commun **296**(2): 343-9.

Ohh, M., C. W. Park, et al. (2000). "Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein." Nat Cell Biol **2**(7): 423-7.

Ohlsson, R., L. Holmgren, et al. (1989). "Insulin-like growth factor 2 and short-range stimulatory loops in control of human placental growth." Embo J **8**(7): 1993-9.

Ohlsson, R., E. Larsson, et al. (1989). "Blastocyst implantation precedes induction of insulin-like growth factor II gene expression in human trophoblasts." Development **106**(3): 555-9.

Okamoto, T. and I. Nishimoto (1991). "Analysis of stimulation-G protein subunit coupling by using active insulin-like growth factor II receptor peptide." Proc Natl Acad Sci U S A **88**(18): 8020-3.

Olson, L. J., R. D. Yammani, et al. (2004). "Structure of uPAR, plasminogen, and sugar-binding sites of the 300 kDa mannose 6-phosphate receptor." Embo J **23**(10): 2019-28.

Page, E. L., G. A. Robitaille, et al. (2002). "Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms." J Biol Chem **277**(50): 48403-9.

Pantaleon, M., H. Jericho, et al. (2003). "The role of insulin-like growth factor II and its receptor in mouse preimplantation development." Reprod Fertil Dev **15**(1): 37-45.

Pantaleon, M. and P. L. Kaye (1996). "IGF-I and insulin regulate glucose transport in mouse blastocysts via IGF-I receptor." Mol Reprod Dev **44**(1): 71-6.

Peng, J., L. Zhang, et al. (2000). "The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodelling." Proc Natl Acad Sci U S A **97**(15): 8386-91.

Peters, T. J., A. Albieri, et al. (1999). "Differentiation-dependent expression of gelatinase B/matrix metalloproteinase-9 in trophoblast cells." Cell Tissue Res **295**(2): 287-96.

Pijnenborg, R., W. B. Robertson, et al. (1981). "Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals." Placenta **2**(1): 71-91.

Pinto, A. B., A. L. Schlein, et al. (2002). "Preimplantation exposure to high insulin-like growth factor I concentrations results in increased resorption rates in vivo." Hum. Reprod. **17**(2): 457-462.

Poikkeus, P., M. Gissler, et al. (2007). "Obstetric and neonatal outcome after single embryo transfer." Hum Reprod **22**(4): 1073-9.

Pugh, C. W., J. F. O'Rourke, et al. (1997). "Activation of Hypoxia-inducible Factor-1; Definition of Regulatory Domains within the alpha Subunit." J. Biol. Chem. **272**(17): 11205-11214.

Qian, D., H.-Y. Lin, et al. (2004). "Normoxic Induction of the Hypoxic-Inducible Factor-1{alpha} by Interleukin-1{beta} Involves the Extracellular Signal-Regulated Kinase 1/2 Pathway in Normal Human Cytotrophoblast Cells." Biol Reprod **70**(6): 1822-1827.

Qiu, Q., A. Basak, et al. (2005). "Role of pro-IGF-II processing by proprotein convertase 4 in human placental development." PNAS **102**(31): 11047-11052.

Rajakumar, A. and K. P. Conrad (2000). "Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta." Biol Reprod **63**(2): 559-69.

Rajakumar, A., K. Doty, et al. (2003). "Impaired Oxygen-dependent Reduction of HIF-1alpha and -2alpha Proteins in Pre-eclamptic Placentae." Placenta **24**(2-3): 199-208.

Rajakumar, A., K. A. Whitelock, et al. (2001). "Selective overexpression of the hypoxia-inducible transcription factor, HIF-2alpha, in placentas from women with preeclampsia." Biol Reprod **64**(2): 499-506.

Rappolee, D. A., K. S. Sturm, et al. (1992). "Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos." Genes Dev **6**(6): 939-52.

Rebourcet, R., F. de Ceuninck, et al. (1998). "Differential distribution of binding sites for 125I-insulin-like growth factor II on trophoblast membranes of human term placenta." Biol Reprod **58**(1): 37-44.

Rechler, M. M. and S. P. Nissley (1985). "The nature and regulation of the receptors for insulin-like growth factors." Annu Rev Physiol **47**: 425-42.

Reddy, U. M., R. J. Wapner, et al. (2007). "Infertility, assisted reproductive technology, and adverse pregnancy outcomes: executive summary of a National Institute of Child Health and Human Development workshop." Obstet Gynecol **109**(4): 967-77.

Redline, R. W., C. L. Chernicky, et al. (1993). "Differential expression of insulin-like growth factor-II in specific regions of the late (post day 9.5) murine placenta." Mol Reprod Dev **36**(2): 121-9.

Richard, D. E., E. Berra, et al. (2000). "Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells." J Biol Chem **275**(35): 26765-71.

Rinderknecht, E. and R. E. Humbel (1978). "Primary structure of human insulin-like growth factor II." FEBS Lett **89**(2): 283-6.

Rinkenberger, J. L., J. C. Cross, et al. (1997). "Molecular genetics of implantation in the mouse." Dev Genet **21**(1): 6-20.

Roberts, C. T., A. N. Sferruzzi-Perri, et al. (2007). "Distinct effects of insulin-like growth factors (IGF) on placental invasion and function." Placenta **28**(8-9): A5; P13.

Rodesch, F., P. Simon, et al. (1992). "Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy." Obstet Gynecol **80**(2): 283-5.

Rosenzweig, S. A. (2004). "What's new in the IGF-binding proteins?" Growth Horm IGF Res **14**(5): 329-36.

Rossant, J. and J. C. Cross (2001). "Placental development: lessons from mouse mutants." Nat Rev Genet **2**(7): 538-48.

Rossignol, F., E. de Laplanche, et al. (2004). "Natural antisense transcripts of HIF-1alpha are conserved in rodents." Gene **339**: 121-30.

Rossignol, F., C. Vache, et al. (2002). "Natural antisense transcripts of hypoxia-inducible factor 1alpha are detected in different normal and tumour human tissues." Gene **299**(1-2): 135-140.

Ryan, H. E., J. Lo, et al. (1998). "HIF-1 alpha is required for solid tumor formation and embryonic vascularization." Embo J **17**(11): 3005-15.

Safran, M. and W. G. Kaelin, Jr. (2003). "HIF hydroxylation and the mammalian oxygen-sensing pathway." J Clin Invest **111**(6): 779-83.

Sagle, M., K. Bishop, et al. (1988). "Recurrent early miscarriage and polycystic ovaries." Bmj **297**(6655): 1027-8.

Salceda, S. and J. Caro (1997). "Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes." J. Biol. Chem. **272**(36): 22642-22647.

Sappino, A. P., J. Huarte, et al. (1989). "Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos." J. Cell Biol. **109**(5): 2471-2479.

Sara, V. R. and K. Hall (1990). "Insulin-like growth factors and their binding proteins." Physiol. Rev. **70**(3): 591-614.

Sato, M., T. Tanaka, et al. (2002). "Inducible Expression of Endothelial PAS Domain Protein-1 by Hypoxia in Human Lung Adenocarcinoma A549 Cells . Role of Src Family Kinases-dependent Pathway." Am. J. Respir. Cell Mol. Biol. **26**(1): 127-134.

Sato, M., T. Tanaka, et al. (2002). "Inducible Expression of Endothelial PAS Domain Protein-1 by Hypoxia in Human Lung Adenocarcinoma A549 Cells. Role of Src Family Kinases-dependent Pathway." Am. J. Respir. Cell Mol. Biol. **26**(1): 127-134.

Schaffer, L., J. Vogel, et al. (2005). "Preserved placental oxygenation and development during severe systemic hypoxia." Am J Physiol Regul Integr Comp Physiol: 00237.2005.

Schultz, G. A., A. Hahnel, et al. (1993). "Expression of IGF ligand and receptor genes during preimplantation mammalian development." Mol Reprod Dev **35**(4): 414-20.

Schultz, G. A., A. Hogan, et al. (1992). "Insulin, insulin-like growth factors and glucose transporters: temporal patterns of gene expression in early murine and bovine embryos." Reprod Fertil Dev **4**(4): 361-71.

Semenza, G. L. (2000). "HIF-1: mediator of physiological and pathophysiological responses to hypoxia." J Appl Physiol **88**(4): 1474-80.

Shevell, T., F. D. Malone, et al. (2005). "Assisted reproductive technology and pregnancy outcome." Obstet Gynecol **106**(5 Pt 1): 1039-45.

Shin, J. C., J. H. Lee, et al. (2003). "Expression of insulin-like growth factor-II and insulin-like growth factor binding protein-1 in the placental basal plate from pre-eclamptic pregnancies." Int J Gynaecol Obstet **81**(3): 273-80.

Shweiki, D., A. Itin, et al. (1993). "Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis." J Clin Invest **91**(5): 2235-43.

Sibley, C. P., P. M. Coan, et al. (2004). "Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta." Proc Natl Acad Sci U S A **101**(21): 8204-8.

Slater, M. and C. R. Murphy (1999). "Differential expression of insulin-like growth factors in the uterine epithelium and extracellular matrix during early pregnancy." Matrix Biol **18**(6): 579-84.

Smith, G. C., J. A. Crossley, et al. (2004). "First-trimester placentation and the risk of antepartum stillbirth." Jama **292**(18): 2249-54.

Solberg, H., J. Rinkenberger, et al. (2003). "A functional overlap of plasminogen and MMPs regulates vascularization during placental development." Development **130**(18): 4439-50.

Soos, M. A., C. E. Field, et al. (1993). "Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity." Biochem J **290 (Pt 2)**: 419-26.

Spinella, F., L. Rosano, et al. (2002). "Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells." J Biol Chem **277**(31): 27850-5.

Srinivas, V., L. P. Zhang, et al. (1999). "Characterization of an oxygen/redox-dependent degradation domain of hypoxia-inducible factor alpha (HIF-alpha) proteins." Biochem Biophys Res Commun **260**(2): 557-61.

Stoeltzing, O., W. Liu, et al. (2003). "Regulation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and angiogenesis by an insulin-like growth

factor-I receptor autocrine loop in human pancreatic cancer." Am J Pathol **163**(3): 1001-11.

Stojanov, T., S. Alechna, et al. (1999). "In-vitro fertilization and culture of mouse embryos in vitro significantly retards the onset of insulin-like growth factor-II expression from the zygotic genome." Mol Hum Reprod **5**(2): 116-24.

Surwit, R. S., C. M. Kuhn, et al. (1988). "Diet-induced type II diabetes in C57BL/6J mice." Diabetes **37**(9): 1163-7.

Takeda, K., V. C. Ho, et al. (2006). "Placental but Not Heart Defects Are Associated with Elevated Hypoxia-Inducible Factor {alpha} Levels in Mice Lacking Prolyl Hydroxylase Domain Protein 2." Mol. Cell. Biol. **26**(22): 8336-8346.

Tanimoto, K., Y. Makino, et al. (2000). "Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein." Embo J **19**(16): 4298-309.

Teesalu, T., F. Blasi, et al. (1996). "Embryo implantation in mouse: fetomaternal coordination in the pattern of expression of uPA, uPAR, PAI-1 and alpha 2MR/LRP genes." Mech Dev **56**(1-2): 103-16.

Teesalu, T., F. Blasi, et al. (1998). "Expression and function of the urokinase type plasminogen activator during mouse hemochorial placental development." Dev Dyn **213**(1): 27-38.

Teesalu, T., R. Masson, et al. (1999). "Expression of matrix metalloproteinases during murine chorioallantoic placenta maturation." Developmental Dynamics **214**(3): 248-258.

Theiler, K. (1972). The house mouse: development and normal stages from fertilisation to 4 weeks of age, Springer-Verlag New York Inc.

Thrash-Bingham, C. A. and K. D. Tartof (1999). "aHIF: a natural antisense transcript overexpressed in human renal cancer and during hypoxia." J Natl Cancer Inst **91**(2): 143-51.

Tian, H., S. L. McKnight, et al. (1997). "Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells." Genes Dev **11**(1): 72-82.

Toye, A. A., J. D. Lippiat, et al. (2005). "A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice." Diabetologia **48**(4): 675-86.

Treins, C., S. Giorgetti-Peraldi, et al. (2002). "Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway." J Biol Chem **277**(31): 27975-81.

Tulppala, M., U. H. Stenman, et al. (1993). "Polycystic ovaries and levels of gonadotrophins and androgens in recurrent miscarriage: prospective study in 50 women." Br J Obstet Gynaecol **100**(4): 348-52.

Tycko, B. and A. Efstratiadis (2002). "Genomic imprinting: piece of cake." Nature **417**(6892): 913-4.

Uchida, T., F. Rossignol, et al. (2004). "Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha." J Biol Chem **279**(15): 14871-8.

Varia, M. A., D. P. Calkins-Adams, et al. (1998). "Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma." Gynecol Oncol **71**(2): 270-7.

Vicovac, L. and J. D. Aplin (1996). "Epithelial-mesenchymal transition during trophoblast differentiation." Acta Anat (Basel) **156**(3): 202-16.

Volpert, O., D. Jackson, et al. (1996). "The insulin-like growth factor II/mannose 6-phosphate receptor is required for proliferin-induced angiogenesis." Endocrinology **137**(9): 3871-6.

Wang, G. L., B. Jiang, et al. (1995). "Hypoxia-Inducible Factor 1 is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O_2 Tension." PNAS **92**(12): 5510-5514.

Wang, G. L. and G. L. Semenza (1995). "Purification and Characterization of Hypoxia-inducible Factor 1." J. Biol. Chem. **270**(3): 1230-1237.

Watson, E. D. and J. C. Cross (2005). "Development of structures and transport functions in the mouse placenta." Physiology (Bethesda) **20**: 180-93.

Wenger, R. and M. Gassmann (1999). HIF-1 and the molecular response to hypoxia in mammals. Environmental stress and gene regulation. K. Storey. Washington DC, BIOS scientific publishers**:** 25-40.

Wenger, R. H., A. Rolfs, et al. (1997). "The mouse gene for hypoxia-inducible factor-1alpha--genomic organization, expression and characterization of an alternative first exon and 5' flanking sequence." Eur J Biochem **246**(1): 155-65.

Wenger, R. H., A. Rolfs, et al. (1996). "Nucleotide Sequence, Chromosomal Assignment and mRNA Expression of Mouse Hypoxia-Inducible Factor-1[alpha]." Biochemical and Biophysical Research Communications **223**(1): 54-59.

Wenger, R. H., A. Rolfs, et al. (1998). "Mouse hypoxia-inducible factor-1alpha is encoded by two different mRNA isoforms: expression from a tissue-specific and a housekeeping-type promoter." Blood **91**(9): 3471-80.

White, C. A., M. Johansson, et al. (2004). "Effect of interleukin-10 null mutation on maternal immune response and reproductive outcome in mice." Biol Reprod **70**(1): 123-31.

Whiteside, E. J., M. M. Jackson, et al. (2001). "Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 are key regulators of extracellular matrix degradation by mouse embryos." Biol Reprod **64**(5): 1331-7.

Wiener, C. M., G. Booth, et al. (1996). "In Vivo Expression of mRNAs Encoding Hypoxia-Inducible Factor 1." Biochemical and Biophysical Research Communications **225**(2): 485-488.

Wiesener, M. S., H. Turley, et al. (1998). "Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha." Blood **92**(7): 2260-8.

Wilson, D. M., A. Bennett, et al. (1982). "Somatomedins in pregnancy: a cross-sectional study of insulin-like growth factors I and II and somatomedin peptide content in normal human pregnancies." J Clin Endocrinol Metab **55**(5): 858-61.

Yagel, S., R. S. Parhar, et al. (1988). "Normal nonmetastatic human trophoblast cells share in vitro invasive properties of malignant cells." J Cell Physiol **136**(3): 455-62.

Yebra, M., G. C. Parry, et al. (1996). "Requirement of receptor-bound urokinase-type plasminogen activator for integrin alphavbeta5-directed cell migration." J Biol Chem **271**(46): 29393-9.

Zelzer, E., Y. Levy, et al. (1998). "Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT." Embo J **17**(17): 5085-94.

Zhang, Q., Y. Wu, et al. (2004). "Crosstalk of hypoxia-mediated signaling pathways in upregulating plasminogen activator inhibitor-1 expression in keloid fibroblasts." J Cell Physiol **199**(1): 89-97.

Zhang, X., G. M. Kidder, et al. (1994). "Expression of plasminogen activator genes and enzymatic activities in rat preimplantation embryos." J Reprod Fertil **101**(1): 235-40.

Zhou, Y., O. Genbacev, et al. (1998). "Oxygen regulates human cytotrophoblast differentiation and invasion: implications for endovascular invasion in normal pregnancy and in pre-eclampsia." J Reprod Immunol **39**(1-2): 197-213.

Zini, J. M., S. C. Murray, et al. (1992). "Characterization of urokinase receptor expression by human placental trophoblasts." Blood **79**(11): 2917-29.

Chapter 7
Appendix: Publications arising from this thesis

Pringle, K.G. and Roberts,C.T. (2007) New light on early post-implantation pregnancy in the mouse: roles for insulin-like growth factor-II (IGF-II)? *Placenta, v.28 (4), pp. 286-297, April 2007*

Pringle, K.G., Kind, K.L., Thompson, J.G. and Roberts, C.T. (2008) Complex Interactions Between Hypoxia Inducible Factors, Insulin-Like Growth Factor-II and Oxygen in Early Murine Trophoblast
Placenta, v.28 (11/12), pp. 1147-1157, November/December 2007