

# THE PIVOTAL ROLE OF INSULIN-LIKE GROWTH FACTORS IN PREGNANCY SUCCESS

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"Opportunity is missed by most people because it is dressed in overalls and looks like work." Thomas Edison

# Abstract

Appropriate placental development in early gestation is essential for subsequent placental function and hence optimal fetal growth and pregnancy outcome. Placental insufficiency has been implicated in common disorders of pregnancy, which result in fetal and maternal mortality or morbidity, and also increase the risk of poor health in adult offspring.

Prior to the onset of maternal blood flow to the placenta at ~10 weeks of gestation, placentation occurs in a relatively hypoxic environment, which is essential for healthy pregnancy. IGF-II is abundantly expressed by the invasive trophoblast and may interact with oxygen to regulate placentation. Additionally, maternally-derived IGFs may act on the placenta and the mother to regulate fetal growth. This thesis investigated the role and interaction of oxygen and IGF-II on human placental outgrowth during early pregnancy *in vitro*. Furthermore, the impact of maternal IGF treatment during early to mid pregnancy, on placental development and substrate transfer, nutrient partitioning between the mother and fetus, and fetal growth, were also determined in mid and late gestation in guinea pigs.

We have demonstrated, using human early first trimester placental villous explants, that IGF-II mediates the effect of hypoxia on placental outgrowth. Culture of placental explants in hypoxia, or with exogenous IGF-II, enhanced trophoblast outgrowth and inhibited TGF- $\beta$ 1 activation, a negative regulator of trophoblast function. In addition, culture of explants in hypoxia induced *Igf2* gene expression in outgrowing trophoblast, without altering *Upar*, *Igf1r*, *Igf2r* or *Tgf $\beta$ 1* transcription. We propose that this novel interaction of oxygen, IGF-II and TGF- $\beta$ 1 during pregnancy is an important determinant of placental development. Furthermore, we showed that exogenous IGF-II stimulates villous explant trophoblast outgrowth in placenta from >10 weeks gestation, suggesting that IGF-II may be a potential therapeutic agent to enhance placental growth.

In guinea pigs, maternal treatment with IGF-I or IGF-II, in early to mid pregnancy, has sustained anabolic effects on fetal growth, enhanced fetal survival and increased placental delivery, and fetal and maternal utilization of, glucose and amino acids near term. These effects were also evident by mid gestation following earlier IGF-I treatment. Despite these similar pregnancy outcomes, there were IGF specific effects on the placenta and mother, suggesting that IGFs may mediate some of their effects via different pathways. IGF-I administration severely reduced maternal adiposity in late pregnancy,

suggesting persistent diversion of nutrients to the fetus. In contrast, IGF-II elicited its effects by substantially improving development of the placental exchange region, which correlated with placental function.

We have suggested that the discrete effects of IGF-I and IGF-II stem from distinct interactions of the IGFs with various receptors. Maternal administration of an analogue of IGF-II that selectively interacts with IGF2R (Leu<sup>27</sup>-IGF-II), revealed that many of the effects of IGF-II treatment, were mediated by IGF2R, while IGF-I presumably acts through IGF1R.

Together, this work has highlighted the major and somewhat complementary roles of maternal IGFs during the first half of pregnancy, in regulating placental development, fetal growth and pregnancy success. Importantly, it indicates the potential use of maternal IGFs in diagnostic and therapeutic approaches to pregnancy complications.

# 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.

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## Publications arising from this thesis

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7. Pringle KG, **Sferruzzi-Perri AN**, Kind KL, Thompson JG, Roberts CT. Control of placental development by hypoxia inducible factors. *In preparation*
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# Abstracts arising from this thesis

2006

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4. Standen P, Lumbers ER, Kumarasamy V, Sferruzzi-Perri AN, Taylor RL, Heinemann G, Owens JA, Roberts CT *Novel interactions of endocrine IGFs with the placental RAS*. Fetal and Neonatal Physiology Workshop, Rottnest Island, Western Australia.
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2005

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14. **Owens JA**, Roberts CT, **Sferruzzi-Perri AN**, Grant P, Robinson JS *Small for gestational age 2005: Understanding the biology and therapeutic consequences*. European Society for Paediatric Endocrinology, Satellite Meeting, Montreux, Switzerland.

## 2004

15. **Sferruzzi-Perri AN**, Owens JA, Robinson JS and Roberts CT *Insulin-like growth factor treatment of pregnant guinea pigs during early pregnancy promotes fetal growth*. National Society for Reproductive Biology Conference, Sydney, Australia.
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19. **Roberts CT**, Grant PA and **Sferruzzi-Perri AN** *IGF-II and hypoxia inhibit activation of latent TGF $\beta$ 1 by IGF2R in first trimester human placenta and thereby control placental differentiation*. Growth Hormone-Insulin-Like Growth Factor, International Symposium, Cairns, Queensland, Australia. Abstract P88.
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21. Sferruzzi-Perri AN and Roberts CT *IGF-2 mediates the effect of hypoxia on human cytotrophoblast outgrowth*. Society for Reproductive Biology, National Conference, Melbourne, Australia. Abstract 87.
22. Sferruzzi-Perri AN and Roberts CT *Hypoxia and IGF-2 promote human cytotrophoblast outgrowth in vitro*. Australian Society for Medical Research, Local Meeting, Adelaide, Australia. Abstract O23.
23. Roberts CT, Sferruzzi-Perri AN, Bussemaker L, Grant PA *Exogenous IGF-2 in early pregnancy enhances fetal and placental growth in the mouse*. 7<sup>th</sup> Perinatal Society of Australia and New Zealand, Annual Congress, Hobart, Australia. Abstract A100.
24. Roberts CT, Sferruzzi-Perri AN, Donaldson AC, Grant PA *IGF-II regulates activation of TGF $\beta$ 1 at the cell surface and thereby controls differentiation of the placenta*. National Australian Society for Medical Research Conference, Adelaide, Australia. Abstract 57.
25. Roberts CT, Sferruzzi-Perri AN, Grant P, Donaldson A & Khong TY *Regulation of placental differentiation by insulin-like growth factor II (IGF-II)* Early Origins of Adult Disease Symposium, Adelaide, Australia.

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# Abbreviations

A	Adenosine
AA	Amino acids
Ang2	Angiopoietin 2
AIB	Methyl [ <sup>14</sup> C]-amino-isobutyric acid
ALS	Acid liable subunit
BM	Basal membrane
ARNT	Arylhydrocarbon receptor nuclear transferase
BM	Basal syncytiotrophoblast membrane
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
cDNA	Complimentary DNA
Chol	Cholesterol
Ct	Cycle threshold
CTB	Cytotrophoblast
Cpm	Counts per minute
DAB	3,3-diaminobenzidine
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EVT	Extravillous cytotrophoblast
FAK	Focal adhesion kinase
FC	Fetal capillary
FFA	Free fatty acids
fwd	Forward primer
hCG	Human chorionic gonadotrophin
hPL	Human placental lactogen
HPLC	High pressure liquid chromatography
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen

HRE	Hypoxia response element
HRP	Horse radish peroxidase
G	Guanine
Gluc	Glucose
Glut	Glucose transporter
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF1R	Type 1 IGF receptor
IGF2R	Type 2 IGF receptor
InsR	Insulin receptor
IUGR	Intrauterine growth restriction
IVS	Intervillous space
kDa	Kilo Daltons
LAP	Latency associated peptide
Leu <sup>27</sup> -IGF-II	[Leu <sup>27</sup> ]-IGF-II
mA	Milli Amps
MBS	Maternal blood space
MG	[ <sup>3</sup> H]-methyl-D-glucose
MHC	Major compatibility complex
MMP	Matrix metalloproteinase
MVM	Microvillous apical syncytiotrophoblast membrane
M6P	Mannose 6 phosphate
mRNA	Messenger RNA
mmHg	Millimetres of mercury
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PIGF	Placental growth factor
rev	Reverse primer
RGD	Arg-Gly-Leu
RIA	Radioimmunoassay
RNA	Ribonucleic acid

Rpm	Revolutions per minute
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulphate
STB	Syncytiotrophoblast
T	Thymine
TIMP	Tissue inhibitors of matrix metalloproteinase
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Trig	Triglycerides
Troph	Trophoblast
T $\beta$ R-V	Type V transforming growth factor- $\beta$ receptor
TGF $\beta$ IR	TGF $\beta$ type 1 receptor
TGF $\beta$ IIR	TGF $\beta$ type 2 receptor
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
V	Volts
VEGF	Vascular endothelial growth factor
VIA	Video image analysis

# *Chapter 1 : Introduction*

## 1.1 Determinants of pregnancy success

During pregnancy it is necessary to ensure adequate nutrient allocation to the fetus, while providing sufficient substrate is also partitioned to the mother to meet the energy requirements needed to maintain maternal health and her capacity to support the conceptus. To ensure an adequate flow of oxygen and nutrients to the developing conceptus, during normal pregnancy, the mother undergoes a number of physiological, "adaptive" changes, which in turn are influenced by factors including genetics, nutrition, lifestyle and hormonal milieu. The placenta, is central to these processes, as it is not only the organ responsible for the exchange of substrates between the mother and fetus, but also synthesises a number of steroid and peptide hormones, secreted into the maternal circulation, that modulate maternal physiology and adaptation to pregnancy.

## 1.2 Maternal adaptation to pregnancy

Alterations in the regulation of maternal appetite, body composition, cardiovascular function, energy consumption and metabolism occur during pregnancy, which ensure an adequate flow of nutrients, oxygen and water to the developing fetus (Butte 2005; Owens 1991). Furthermore, despite the mother eating intermittently during the day, throughout pregnancy she must continuously supply nutrients to the fetus to sustain its exponential growth (Butte 2000; Hytten and Leich 1971). To achieve this, the mother changes her metabolism of fat and glucose and acquires a state of insulin resistance. At the same time, women increase their basal hepatic glucose production during pregnancy by 16-30% (Assel, Rossi *et al.* 1993; Catalano, Roman-Drago *et al.* 1998; Catalano, Tyzbir *et al.* 1992), while increasing their daily rate of carbohydrate utilization by 34% (Butte 2000), to meet the demands of the conceptus. Despite increased carbohydrate utilization, insulin-stimulated glucose uptake in late pregnancy is 50-70% lower than that of non-pregnant women (Catalano, Tyzbir *et al.* 1991; Catalano, Tyzbir *et al.* 1993; Catalano, Tyzbir *et al.* 1992; Ryan, O'Sullivan *et al.* 1985), suggesting diminished maternal peripheral insulin sensitivity. The development of relative insulin resistance during pregnancy is thought to be due to inhibition of post-receptor insulin signaling as pregnant women displayed reduced phosphorylation and activity of the insulin receptor tyrosine kinase in skeletal muscle (rectus abdominus), compared with non-pregnant controls (Shao, Catalano *et al.* 2000). This increased insulin resistance reduces maternal glucose utilization, sparing carbohydrates for the rapidly growing fetus.

Placental elaboration of hormones, including estrogen, prolactin, progestins, which are released into the circulation of the mother are thought to contribute to the observed maternal metabolic changes (Butte

2000; Catalano 1999; Ryan and Enns 1988). Certainly, changes in glucose tolerance and  $\beta$ -cell responsiveness to glucose during pregnancy coincide with increases in circulating cortisol, prolactin, placental lactogen, estrogen, and progesterone levels, which all have been shown to modulate glucose metabolism and insulin action (Butte 2000; Picard, Wanatabe *et al.* 2002). Therefore the placenta determines nutrient delivery to the fetus both directly as the organ responsible for their transfer, and indirectly, as a major source of endocrine modulators of maternal metabolism to increase their availability.

## 1.3 Placenta

### 1.3.1 Introduction

The placenta develops during pregnancy and is the functional interface between the mother and fetus. During pregnancy, the placenta performs six essential functions that ensure a suitable environment for optimal fetal development. The placenta 1) anchors the conceptus to the maternal endometrium, 2) supplies the developing fetus with oxygen and nutrients, transporting them from the mother into the umbilical circulation whilst removing waste products, 3) prevents immunological rejection of the semi-allogeneic conceptus by the maternal immune system, 4) synthesises an array of hormones, cytokines, growth factors and other bioactive molecules critical to the maintenance of pregnancy and preparation for lactation, 5) enables passive immunity for the fetus through the transport of maternal antibodies and 6) acts as a barrier to pathogens (Bauer, Harding *et al.* 1998; Cross, Werb *et al.* 1994; Gude, Roberts *et al.* 2004; Redman, Sargent *et al.* 1993; Robinson, Chidzanja *et al.* 1995; Robinson, Seamark *et al.* 1994). Through these functions, the placenta is able to modulate the maternal environment to support normal fetal development, as well as maintaining the health of the mother.

The process of placental development is dynamic and highly regulated. It involves an orchestrated balance of placental trophoblast cell proliferation, differentiation and invasion. Placentation is initiated at the time of implantation when the trophoectoderm layer surrounding the blastocyst makes contact with the uterine wall. During pregnancy, trophoblasts invade and remodel the uterine endometrium and its vasculature, forming a feto-maternal interface with access to the maternal circulation. This process is crucial for efficient exchange of oxygen and nutrients. The highly proliferative, invasive, lack of cell contact inhibition and immune privilege properties of trophoblasts are reminiscent of a metastasising cancer. However, during normal pregnancy, trophoblast behaviour is tightly regulated, such that

trophoblast invasion remains confined to the first third of the myometrium and continues only until about the middle of pregnancy.

### ***1.3.2 Human placentation in early pregnancy***

#### ***1.3.2.1 Blastocyst apposition, attachment and implantation***

In humans, at around 5 to 6 days post-fertilisation, the blastocyst attaches to the uterine epithelium at the embryonic pole. At this time the trophoblast layer of the blastocyst in contact with the epithelium rapidly proliferates and divides into two trophoblast layers (Figure 1.1a). Some of these proliferating cells called cytotrophoblasts (CTBs), lose their cell membranes and fuse to form a multinucleated cytoplasmic mass known as the syncytiotrophoblast (STB) (Benirschke 1990). Those cells that constitute the blastocyst wall and do not lose their cell membranes form the CTB layer. Finger-like projections of the STB intrude through the luminal epithelium eroding and invading the underlying endometrial stroma by degrading local extracellular matrix (Pijnenborg, Robertson *et al.* 1981), enabling the blastocyst to progressively implant into the maternal endometrium (Figure 1.1a).

#### ***1.3.2.2 Formation of placental lacunar networks***

At around 9-10 days, isolated spaces known as lacunae begin to appear within the STB and maternal capillaries around the implanted embryo expand and become dilated to form sinusoids (Benirschke 1990; Fox 1989; Moore 1974; Redman, Sargent *et al.* 1993) (Figure 1.1b). As the STB moves further into the uterine endometrium, more lacunae appear, and those near each other fuse to form lacunar networks, which are the precursors of placental intervillous spaces (Burton, Jauniaux *et al.* 1999) (Figure 1.1c)

#### ***1.3.2.3 The formation of placental villi***

Between the 11<sup>th</sup> and 13<sup>th</sup> day, cytotrophoblasts beneath the STB proliferate to form local masses that evaginate into the STB mass, forming the primary stem villi (Benirschke 1990; Fox 1989; Moore 1974; Redman, Sargent *et al.* 1993) (Figure 1.1d). Some of the CTB within the primary villus fuse with the overlying syncytium and lose their cell membranes to enable further expansion of the multinucleated mass. Extension of the CTB core allows the villus to push into blood-filled lacunae, forming intervillous spaces.

Shortly after primary villi appear, they begin to branch and by days 15 to 16 of pregnancy the extraembryonic mesenchyme associated with the allantois (an evagination of hindgut region of the

**Figure 1.1 Schematic representation of human placental development during the first trimester.**

On gestational days 5 to 6, the blastocyst contacts the uterine epithelium at the embryonic pole, inducing trophoblast to proliferate and differentiate into syncytiotrophoblast (STB) and cytotrophoblast (CTB) (A). Expansion of the STB allows the blastocyst to slowly embed within the uterine endometrium. By day 9, lacunae begin to appear within the STB and maternal capillaries around the implanting embryo expand and become dilated to form sinusoids (B). By 10 days, the embryo is completely implanted within the endometrium and lacunae fuse with those adjacent to form lacunar networks, which are the precursor of placental intervillous spaces (C). Between days 11 and 13, primary stem villi form by the proliferation of CTBs beneath the STB (D). By days 15 to 16 of pregnancy the extraembryonic mesenchyme lying beneath the CTB grows into the core of each primary stem villus, forming secondary stem villi (depicted in cross-section) (E). Villous mesenchymal cells differentiate into fetal blood capillaries from day 21 of gestation, which connect to the embryo and form tertiary stem villi (seen in cross-section) (F). Modified from Moore, 1974.

NOTE: This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.



embryo) and lying beneath the CTB proliferates and grows into the centre of each primary stem villus, forming secondary stem villi (Benirschke 1990; Fox 1989; Moore 1974; Redman, Sargent *et al.* 1993) (Figure 1.1e). By 21 days of gestation, the mesenchymal cells in the villi differentiate into connective tissue and fetal blood capillaries that connect to the embryo, establishing the uteroplacental circulation and creating tertiary stem villi (King 1987) (Figure 1.1f).

#### 1.3.2.4 Trophoblast differentiation

CTB of the tertiary villi may enter the villous or extravillous pathways. In villi that float in the intervillous space, CTBs progress along the villous pathway proliferate and fuse with the overlying syncytium, which mediates nutrient and gas exchange for the developing fetus. CTBs at the tips of villi in contact with the decidua proliferate, penetrate and extend through the overlying villous syncytium allowing contact to be made with the maternal tissue and proceed along the extravillous pathway (Pijnenborg, Bland *et al.* 1981; Pijnenborg, Robertson *et al.* 1981). These CTB extensions form cell columns that aggregate with adjacent columns to form the cytotrophoblastic shell, which constitutes the maternal-fetal interface and encompasses the entire conceptus. Columns of CTB anchor the tips of villi to the maternal decidual stroma and are thought to play a role in the structural integrity of the maternal-fetal interface.

#### 1.3.2.5 Extravillous cytotrophoblast

From anchoring villi, invasive CTBs, called extravillous cytotrophoblasts (EVTs) detach from cell columns individually or in small clusters called CTB islands and colonize the maternal interstitium and vasculature, termed interstitial and endovascular invasion, respectively (Burton, Jauniaux *et al.* 1999; Pijnenborg, Bland *et al.* 1983; Pijnenborg, Bland *et al.* 1981; Redman, Sargent *et al.* 1993) (Figure 1.2). To become extravillous, CTBs undergo an "epithelial to mesenchymal transformation" (see below). This invasive process results in the circumferential expansion of the placenta therefore increasing the surface area for exchange between the mother and the fetus and dilated maternal arterioles, which are no longer responsive to maternal vasocontrol and promotes blood flow to the placenta (Kam, Gardner *et al.* 1999; Pijnenborg, Dixon *et al.* 1980; Pijnenborg, Vercruyssen *et al.* 2006; Pijnenborg, Vercruyssen *et al.* 1998). Virtually all of the 100-150 maternal spiral arteries in the placental bed are invaded and transformed into distended vessels with diameters of 200-300  $\mu\text{m}$  (Lyll 2005) that promote blood flow to the placenta, which constitutes 30% of the maternal cardiac output by term (Khong, De Wolf *et al.* 1986; Martin 1968)

NOTE: This figure is included on page 7 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.2 Schematic representation of the feto-maternal interface during the first trimester in human pregnancy.**

From tips of anchoring villi (AV), CTB proliferate and form cell columns (CC), which make direct contact with the maternal decidua. Column CTB (cCTB) proliferate and differentiate into extravillous cytotrophoblast (EVTs), which invade the maternal interstitium and vasculature. Also depicted in this figure, is a floating villus, which floats in the intervillous space and mediates nutrient and gas exchange for the developing fetus.

Modified from Genbacev *et al.*, 2001.

#### 1.3.2.5.1 *Interstitial invasion*

Interstitial EVT<sub>s</sub> invade the decidual stroma and reach the endometrial/myometrium boundary by the eighth week of gestation (Pijnenborg, Bland *et al.* 1981). Subsequently, they invade deeper into the decidua, reaching the first third of the myometrium and concentrate around maternal spiral arteries (Pijnenborg, Bland *et al.* 1983). Interstitial CTBs surrounding spiral arteries have been suggested to “prepare” them for endovascular trophoblast migration by initiating medial necrosis and fibrinoid deposition in the spiral arterioles (Kam, Gardner *et al.* 1999) and release of vasoactive agents such as carbon monoxide (Lyall 2003). Terminal differentiation of interstitial EVT<sub>s</sub> occurs when they fuse to form the non-invasive, multinuclear placental bed giant cells (Pijnenborg 1996).

#### 1.3.2.5.2 *Endovascular invasion*

Endovascular invasion commences at least as early as 4-6 weeks of gestation, where myometrial arteries are reached by 10 weeks of gestation (Pijnenborg, Bland *et al.* 1983) and may migrate as far as the first third of the myometrium (Brosens 1975; Brosens, Robertson *et al.* 1967). EVT<sub>s</sub> become endovascular when they enter the vessel lumen by penetration of, and transmigration through, the endothelium (Kam, Gardner *et al.* 1999). Following EVT invasion, EVT<sub>s</sub> can then also enter the vessel lumen by migrating from the cytotrophoblastic shell along the inner walls of the maternal spiral arterioles that have become continuous with IVS (Kam, Gardner *et al.* 1999).

Within the spiral arterioles, endovascular EVT<sub>s</sub> progressively replace the vascular endothelial cells, the smooth muscle layer and deposit a fibrinoid matrix, as well as replacing the neural tissue in myometrial segments of arteries (Aplin 1991; Pijnenborg, Bland *et al.* 1983). This transforms the narrow spiral arterioles into dilated tubes, unresponsive to vasoconstrictive control and capable of high conductance (Brosens, Robertson *et al.* 1967) (Figure 1.3)

#### 1.3.2.5.3 *Trophoblast epithelial – mesenchymal transformation*

The ability of EVT<sub>s</sub> to invade maternal tissues, involves (1) changes in cell-cell associations, (2) cell adhesion to the ECM, (3) polar degradation of the ECM in the direction of migration and (4) active movement through the matrix. In order to become extravillous and invade, CTBs undergo a temporally and spatially regulated “epithelial to mesenchymal transformation”, losing their intercellular junctions and expressing an altered repertoire of adhesion molecules (Aboagye-Mathiesen, Laugesen *et al.* 1996; Aplin 1991; Damsky, Fitzgerald *et al.* 1992; Damsky, Librach *et al.* 1994; Vicovac and Aplin 1996) (Figure 1.4).

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 Photomicrograph showing the presence of endovascular EVT within maternal spiral arteries during human pregnancy.**

Figure from Grosser, 1924, as reviewed by Pijnenborg *et al.*, 2006.

The villous CTB layer, a polarised highly organised and proliferative epithelium, expresses high levels of laminin and collagen IV, the intercellular binding molecule E-cadherin and also the  $\alpha 6$  and  $\beta 4$  integrins, which hetero-dimerise to form the  $\alpha 6\beta 4$  laminin receptor (Figure 1.4). As cells leave the villous basement membrane to form cell columns, expression of laminin is lost and fibronectin expression is acquired (Damsky, Librach *et al.* 1994). E-cadherin,  $\alpha 6$  and  $\beta 4$  are down-regulated and are accompanied by an upregulation of the  $\alpha 5$  and  $\beta 1$  integrin subunits (Aplin 1997; Aplin, Haigh *et al.* 1999; Damsky, Librach *et al.* 1994) (Figure 1.4). Secretion of matrix metalloproteinase-2 (MMP-2; gelatinase A) allows degradation of the ECM (Bischof, Martelli *et al.* 1995; Bischof, Meisser *et al.* 2000; Huppertz, Kertschanska *et al.* 1998). Expression of the serine protease, urokinase-type plasminogen activator (uPA) activates plasmin, which in turn activates latent MMPs, which enable further degradation of ECM (Chakraborty, Gleeson *et al.* 2002; Floridon, Nielsen *et al.* 1999; Floridon, Nielsen *et al.* 2000). CTB expression of the uPA receptor (uPAR) allows interaction with different integrins ( $\beta 1$ ,  $\beta 2$ ,  $\beta 3$  and  $\beta 5$  integrins subunits and the  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins) that promote cytoskeletal reorganisation (Chapman 1997; Degryse, Orlando *et al.* 2001) and cell migration (Carriero, Del Vecchio *et al.* 1999; Chapman 1997; Degryse, Orlando *et al.* 2001; Wei, Lukashev *et al.* 1996; Xue, Mizukami *et al.* 1997).

In more distal regions of the cell column, CTBs cease proliferation and differentiate further to become more invasive by the induction of  $\alpha 1$  integrin expression allowing CTBs to leave the cell column (Aboagye-Mathiesen, Laugesen *et al.* 1996) (Figure 1.4). The integrin heterodimers,  $\alpha 5\beta 1$  and  $\alpha 1\beta 1$ , allow CTBs to interact with fibronectin and laminins/collagens, respectively, and are characteristic of endothelial integrins. Interaction of CTBs with fibronectin is thought to be involved in anchorage to the decidua while interaction with laminin and collagen is essential for invasion and migration (Damsky, Librach *et al.* 1994). No E-cadherin or  $\alpha 6\beta 4$  expression is evident on the surface of these highly invasive CTBs, permitting the detachment of individual cells (Figure 1.4).

Up-regulation of MMP-9 expression, allows additional digestion of the endometrial ECM (Bischof, Martelli *et al.* 1995; Bischof, Meisser *et al.* 2000; Bischof, Meisser *et al.* 2002; Huppertz, Kertschanska *et al.* 1998; Kemp, Kertschanska *et al.* 2002; Librach, Feigenbaum *et al.* 1994; Librach, Werb *et al.* 1991) (Figure 1.4). Trophoblast interaction with the ECM proteins, such as paxillin and vinculin (Kabir-Salmani, Shiokawa *et al.* 2003) through phosphorylation of the focal adhesion kinase (FAK), which is used to mediate ECM effects on cellular gene expression, is also critical to EVT invasion (Ilic, Genbacev *et al.* 2001). Tissue inhibitors of matrix metalloproteinases (TIMPs) and plasminogen activator inhibitors

NOTE: This figure is included on page 11 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.4 Phenotypic changes that occur along the CTB invasive pathway.**  
Modified from Genbacev *et al.*, 2001. Refer to text for references for flow chart.

(PAIs) are expressed predominantly by the decidua but also by the EVT's themselves to control trophoblast invasiveness.

Trophoblasts also up-regulate HLA-G, a trophoblast-specific HLA class I molecule and L-selectin (Genbacev, Prakobphol *et al.* 2003), during the invasive process. The latter is characteristically expressed by leukocytes and may aid EVT invasion by modulating maternal immune responses during pregnancy and permit their movement through the decidua (McMaster, Librach *et al.* 1995; Paul, Rouas-Freiss *et al.* 2000).

Endovascular EVT's undergo additional changes in their expression of cell surface molecules, which allow them to interact with, and ultimately 'mimic', endothelial cell behaviour (Figure 1.4). Endovascular EVT's express classical endothelial integrins  $\alpha 1\beta 1$  (as described previously), the fibrinogen receptor  $\alpha 4\beta 1$ ,  $\alpha v\beta 3$  (binds to vitronectin and fibronectin) as well as VE-cadherin (vascular-endothelial cadherin), VCAM and PECAM (Zhou, Fisher *et al.* 1997). Furthermore, EVT's secrete vascular endothelial growth factor (VEGF)-C and placental growth factor (PIGF) and express angiopoietin (Ang) 2, factors heavily involved in maternal spiral arterial interaction and remodelling (Zhou, Bellingard *et al.* 2003). Along the invasive pathway, EVT's up-regulate their expression of insulin-like growth factor (IGF)-II (Han, Bassett *et al.* 1996; Ohlsson, Holmgren *et al.* 1989; Ohlsson, Larsson *et al.* 1989), which plays fundamental roles in modulating trophoblast function (see section 1.3.5.1.7.2). EVT's are known to upregulate chemokine receptors as they invade the uterine stroma and vasculature, which is thought to be important for "homing" of trophoblast to the maternal endothelium (Hannan, Jones *et al.* 2006).

#### 1.3.2.5.4 Importance of EVT invasion to pregnancy outcome

Poor invasion of the endometrium by EVT's results in insufficient maternal blood perfusion of the placenta, restricting nutrient supply to the developing conceptus, and is associated with common and major disorders of pregnancy (Figure 1.5). These include, miscarriage (Khong, Liddell *et al.* 1987), intrauterine growth restriction (IUGR) (Khong, De Wolf *et al.* 1986; Khong, Liddell *et al.* 1987), preeclampsia (Khong, De Wolf *et al.* 1986), placental abruption (Dommissse and Tiltman 1992), preterm labour (Kim, Bujold *et al.* 2003; Kim, Chaiworapongsa *et al.* 2002) and stillbirth (Khong, De Wolf *et al.* 1986; Robertson, Brosens *et al.* 1967). Combined, these disorders can lead to fetal and maternal mortality and morbidity, affect more than 1 in 5 first-time pregnancies in Western countries (Laws and Sullivan 2004) and may be higher than 40% in developing nations (Albertsson-Wikland, Wennergren *et al.* 1993). Most significantly, they are currently unpreventable.

NOTE: This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.5 Schematic representation of the process of invasion and remodelling of the maternal spiral arteries by invasive cytotrophoblast (EVTs) during pregnancy.**

In normal pregnancy, EVT's remodel decidual and first third of the myometrial arteries, resulting in dilation of spiral arteries. This accommodates a dramatic increase in maternal blood supply to the uterus that is essential for fetal growth during pregnancy. Insufficient invasion and remodelling of the maternal uterine vasculature can lead to early pregnancy loss (miscarriage) or the development of pregnancy diseases which can lead to fetal and maternal mortality and morbidity. CTB = cytotrophoblast, IUGR = intrauterine growth restriction, IVS = intervillous space, PE = preeclampsia, PTB = preterm birth, STB = syncytiotrophoblast. Modified from Red-Horse et al., 2004



Furthermore, perturbed fetal growth, which is usually diagnosed by reduced weight, length and/or increased thinness for gestational age at birth (Karlberg, Albertsson-Wikland *et al.* 1996; Robinson, Seamark *et al.* 1994) (Figure 1.6), is a significant cause of perinatal morbidity and mortality (Fitzhardinge 1985; Kramer 1990; Kramer, Olivier *et al.* 1990; Low, Handley-Derry *et al.* 1992; Smith, Smith *et al.* 1998) and increases the risk of poor health in childhood and adult life (Barker 1998; Barker 2004a; Barker 2004b; Barker 2004c). This predisposition to adult disease forms the fetal programming hypothesis, which proposes that events that take place in utero can profoundly affect the structure,

function or metabolism of major organs or tissues thus affecting susceptibility to disease later in life (Barker 1995). Specially, IUGR is associated with atherosclerosis (Martyn, Gale *et al.* 1998), coronary heart disease (Barker 1995), hypertension (Barker, Bull *et al.* 1990), insulin resistance, non-insulin dependent diabetes (Barker 1998; Barker and Clark 1997) and elevated plasma cholesterol (Barker, Martyn *et al.* 1993) in the offspring later in adult life.

Based on the growing epidemiological evidence that appropriate EVT invasion and placental function is critical to pregnancy success and in the long-term programming of adult disease, it is important that mechanisms regulating trophoblast differentiation into the extravillous lineage are defined. Certainly, EVTs of preeclamptic pregnancies express high levels of the  $\alpha6\beta4$ ,  $\alpha5\beta1$  and  $\alpha v\beta6$  integrins as well as E-cadherin, fail to switch on  $\alpha1\beta1$ ,  $\alpha v\beta3$ , VE-cadherin, VCAM-1 and PECAM-1 (Zhou, Damsky *et al.* 1993; Zhou, Damsky *et al.* 1997; Zhou, Fisher *et al.* 1997) and express significantly less MMP-2 and MMP-9 (Campbell, Rowe *et al.* 2004), suggesting perturbed CTB epithelial to mesenchymal transformation.

#### 1.3.2.6 Placental transport

Probably the most important and well-known functions of the placenta is its transport of nutrients and oxygen from the maternal circulation to the fetus. The placenta is also a metabolically active organ that extracts approximately 40 to 60% of the total glucose and oxygen supplied by the uterine circulation during pregnancy (Bauer, Harding *et al.* 1998). The remaining nutrients and metabolites are transferred across the placenta to the fetus by nutrient specific processes, including passive diffusion, facilitated diffusion, active transport, endocytosis, or exocytosis, as reviewed by (Murphy, Smith *et al.* 2006).

NOTE: This figure is included on page 15 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.6 Photograph demonstrating the growth of appropriately grown (left) and growth restricted (right) babies at birth.**

The latter have reduced weight, length and/or increased thinness for gestational age.

Photo taken by Robinson, JS.

The primary barrier to substrate transfer in the placenta varies between species. In humans there is only one layer of STB separating the fetal and maternal blood circulations (Figure 1.7). The microvillous apical membrane (MVM) of STB is in direct contact with the maternal blood, whilst the basal cell membrane (BM) faces the fetal capillaries. Nutrients must pass through both membranes for delivery to the fetus. Embedded on the surface of the STB membranes, are carrier molecules responsible for transporting nutrients. The regulation of transporter function is not completely understood however transporters on the two membranes must act in concert to achieve appropriate net transfer of nutrients from mother to fetus. Studies of placental transport, *in vitro*, have employed the use of isolated STB plasma membranes and more recently have measured uptake by human placental villous explants. *In vivo* animal studies have utilized radiolabelled non-metabolisable analogues of specific nutrients, which are administered to the mother prior to death and their subsequent uptake by placental and fetal tissues measured.

#### 1.3.2.6.1 Determinants of transport capacity

As pregnancy advances, the increasing demands of the fetus are met by modulation of placental transport capacity. Such changes include a reduction in the thickness of the syncytiotrophoblast barrier for exchange, increased surface area across which placental transport can occur and placental blood perfusion. Furthermore, permeability of substrate, abundance and localization of transporters at the cell surface and substrate concentration gradient influence transplacental capacity.

Transport by passive diffusion (of oxygen, carbon dioxide and urea) is limited by the placental exchange area and blood flow. Facilitated diffusion involves transfer down a concentration gradient by a carrier molecule, without a requirement for additional energy. Active transport requires both carrier proteins and the input of additional energy (Bauer, Harding *et al.* 1998). Glucose and amino acids are the main substrates utilized by the fetus, and thus we have focused on transport of these nutrients.

#### 1.3.2.6.2 Glucose

Fetal gluconeogenesis is relatively low and thus the fetus is critically dependent on placental delivery of glucose from the mother. Glucose transfer is a facilitated process, dependent on the difference between maternal and fetal circulating glucose concentrations (Schneider, Calderon *et al.* 1981) and is mediated by glucose transporters (GLUTs). The human placenta expresses GLUT-1, GLUT-3 (Hahn, Hartmann *et al.* 1995; Illsley 2000; Jansson, Wennergren *et al.* 1993; Tadokoro, Yoshimoto *et al.* 1996), GLUT-4 (Ericsson, Hamark *et al.* 2005b) and GLUT-12 (Gude, Stevenson *et al.* 2003; Rogers, Macheda *et al.* 2002). Placental glucose transport capacity increases with gestation to meet the increasing demands of

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.7 Representation of the placental barrier for substrate transfer between the maternal and fetal circulations in humans.**

The microvillous apical membrane (MVM) of syncytiotrophoblast is in direct contact with the maternal blood, whilst the basal cell membrane (BM) faces the fetal circulation. The pathway for exchange is denoted by ----- . Illustration from Jansson *et al.*, 2006.

the fetus by increasing *Glut-1* mRNA throughout pregnancy (Sakata, Kurachi *et al.* 1995) and increased placental surface area for exchange from the second half of pregnancy (Teasdale and Jean-Jacques 1985). These gestational changes at the feto-maternal interface result in a 50% increase in the uptake of glucose by the human placenta from 1<sup>st</sup> trimester to term, as measured *in vitro* in villous explants (Ericsson, Hamark *et al.* 2005a).

GLUT-1 is the primary mediator of glucose transfer. In the placenta it is the predominant glucose transporter expressed by the STB in term human placenta and uptake of glucose by the placenta is directly correlated to its abundance (Jansson, Wennergren *et al.* 1993). Other work comparing GLUT-1 density on the MVM and BM isolated from term human placenta have revealed that there is a 3-fold greater distribution of this transporter on the MVM plasma membrane than on the BM (Jansson, Wennergren *et al.* 1993; Tadokoro, Yoshimoto *et al.* 1996; Takata, Kasahara *et al.* 1992). Furthermore, it has been estimated that the glucose transport capacity of the MVM is approximately 20-fold higher than that of BM (Jansson, Wennergren *et al.* 1993). The surface area of MVM is over 5-fold greater than the BM due to the development of microvilli, which is thought to contribute to the asymmetrical expression of GLUT-1 (Teasdale and Jean-Jacques 1985).

Investigators have hypothesized that transport across the BM is the rate-limiting step in placental glucose transport (Ilsley, Hall *et al.* 1986). This high MVM transport capacity may act to maximize the glucose concentration gradient between the STB and fetal plasma (Ilsley 2000) and enhance placental transport of glucose to the fetus. An alternate explanation for the differential expression of GLUT-1 on the MVM and BM may be that the placenta utilizes much of the glucose taken up for its own metabolic purposes and thus less transporter is required on the BM to meet the demands of the fetus. Nevertheless, studies performed on placental samples from women suffering from insulin-dependent diabetes mellitus have indicated that GLUT-1 expression and glucose transport was increased on the BM, without an alteration on MVM (Gaither, Quraishi *et al.* 1999; Jansson, Wennergren *et al.* 1999). Studies performed on placenta from women suffering gestational diabetes have been less conclusive. Gaither and colleagues, demonstrated increased GLUT-1 expression and glucose transport in BM in gestational diabetic women (Gaither, Quraishi *et al.* 1999), whilst others reported no difference (Jansson, Ekstrand *et al.* 2001). Furthermore, there is no difference in GLUT-1 expression and activity in term or preterm human placenta from IUGR pregnancies (Jansson, Wennergren *et al.* 1993; Jansson, Ylven *et al.* 2002; Kainulainen, Jarvinen *et al.* 1997), although the babies are hypoglycemic (Marconi, Paolini *et al.* 1996; Nicolini, Hubinont *et al.* 1989). There is also no effect of experimental induction of IUGR in guinea pigs on glucose transfer by the placenta (Jansson and Persson 1990). The differences

in placental glucose transport effects between studies warrant further investigation, particularly on the control and role of placental glucose transfer in regulating fetal growth. Interestingly, insulin receptors are most abundant on fetal endothelium in placenta, suggesting that the fetus may signal to the placenta to alter its transport function (Hiden, Maier *et al.* 2006). Support for this has been observed in past studies performed in sheep (Harding, Liu *et al.* 1994; Jensen, Harding *et al.* 1999; Owens, Kind *et al.* 1994) and mice (Constancia, Angiolini *et al.* 2005).

#### 1.3.2.6.3 Amino acids

High rates of protein synthesis occur in the fetus. Therefore, placental delivery of amino acids, particularly those that are essential, are required to ensure growth. Amino acids are also potent stimulators of fetal insulin release (Milner and Hill 1984) and it has been estimated that 20-40% of the energy supplied to the fetus, is in the form of amino acids (Bauer, Harding *et al.* 1998). It is not surprising then, that reduced fetal plasma amino acid concentrations are associated with restricted growth at term in humans (Cetin, Corbetta *et al.* 1990). In general, transport of amino acids is an active process, as the concentrations of most amino acids in the fetus are greater than those in the mother (Battaglia 1992; Beckman, Pugarelli *et al.* 1990; Lemons, Adcock *et al.* 1976; Montgomery and Young 1982). However, some amino acids may be transporter via facilitated diffusion (Verrey, Closs *et al.* 2004). There are different families of amino acid transporters defined by the class of amino acids they transport and may have overlapping specificities (Jansson 2001; Regnault, de Vrijer *et al.* 2002b; Regnault, Friedman *et al.* 2005). Transport systems may be coupled to an electrochemical gradient of sodium ions through co-transport of sodium, termed sodium-dependent transporters (systems A, ASC and  $\beta$ ). Conversely, there are also sodium-independent transporters (system L, X<sup>-</sup><sub>AG</sub>, Y<sup>+</sup>, b<sup>0,+</sup> and y<sup>+</sup>L) (Jansson 2001). Based on the central role System A transporters play in fetal growth and metabolism (Regnault, Friedman *et al.* 2005), this system will be discussed.

System A transporters interact with short side-chain neutral amino acids, including glycine, alanine, glutamine and serine (McGivan and Pastor-Anglada 1994), which are generally gluconeogenic. The human placenta has a greater distribution of System A transporters on the MVM than on the BM (Hoeltzli and Smith 1989). Indeed, the MVM displays a 3-fold greater System A transporter activity, compared with BM (Johnson and Smith 1988). This suggests that factors in the maternal circulation have the potential to alter placental amino acid uptake and transfer to the fetus by the regulation of System A on MVM.

MVM vesicles prepared from placentas of small for gestational age babies (Dicke and Henderson 1988; Mahendran, Donnai *et al.* 1993) and IUGR (Glazier, Cetin *et al.* 1997; Jansson, Scholtbach *et al.* 1998; Jansson, Ylven *et al.* 2002) have reduced system A amino acid transporter activity. These are consistent with studies showing reduced placental delivery of maternally-administered radiolabelled amino acids, such as leucine, to the umbilical circulation in women bearing growth restricted babies (Paolini, Marconi *et al.* 2001) and reduced placental System A transport capacity, following experimental induction of IUGR by unilateral uterine arterial ligation in guinea pigs (Jansson and Persson 1990). Indeed, *in vivo* inhibition (by 40%) of System A transport between days 7 and 20 of gestation results in IUGR in rats near term (Cramer, Beveridge *et al.* 2002). Recent work performed in pregnant rats fed a low protein diet revealed that reductions in placental System A transporter gene expression and activity occurred prior to the onset of fetal growth restriction (Jansson, Pettersson *et al.* 2006). These studies provide evidence that impaired placental amino acid transporter activity restricts amino acid supply to the fetus and may contribute to reduced fetal growth.

The activity of System A transporters is also impaired in women suffering metabolic disease during pregnancy. For instance, cultured placental MVM from human gestational diabetic and insulin dependent diabetes mellitus women display increased System A transport activity (Jansson, Ekstrand *et al.* 2002). However, this is in contrast to findings of another research group that reported reduced System A transport in pregnancies complicated by insulin dependent diabetes mellitus, which result in macrosomia (Kuruville, D'Souza *et al.* 1994). Inconsistencies may reflect differences in populations sampled, as there was increased placental weight in the former study (Jansson, Ekstrand *et al.* 2002) and no effect on placental weight in the latter study (Kuruville, D'Souza *et al.* 1994). Nevertheless, System A transporters clearly play dominant roles in the regulation of fetal growth and therefore, a better understanding of their regulation may help to understand the pathogenesis of pregnancy disorders including IUGR.

### ***1.3.3 In vitro study of placentation***

There is conflicting data regarding the control of trophoblast proliferation versus differentiation along the invasive pathway, *in vitro*. This may be explained by different methodologies used by investigators and therefore should be considered before making conclusions. For instance, when comparing studies that use different trophoblast cell lines, the source of the cell lines (normal or malignant tissue and first trimester or term placenta), how they were isolated, the degree of differentiation of the cell line and validity of the criteria used to characterise the cell line as being villous or extravillous CTBs all should be

considered (Blaschitz, Hutter *et al.* 2000; Frank, Genbacev *et al.* 2000; King, Thomas *et al.* 2000; Ma, Yang *et al.* 2001; Shiverick, King *et al.* 2001). The same concerns exist when investigators use isolated trophoblast subpopulations prepared from one placenta, or generated from pools of placentae.

The development of the human placental villous explant system *in vitro* in the mid 1980s (Fisher, Cui *et al.* 1989; Fisher, Leitch *et al.* 1985) enabled the basic architecture of placental tissue at anchorage sites to be maintained allowing mechanisms of EVT function and regulation to be investigated. This is important as cell-matrix and cell-cell interactions are known to be critical modulators of autocrine and paracrine actions of a variety of molecules. In 1985 and 1989, Fisher and associates reported that human placental villi cultured on an extracellular matrix (produced by specific cell lines) promoted focal attachment and subsequent outgrowth and migration of CTBs from the tips of villi by digestion of the matrix (Fisher, Cui *et al.* 1989; Fisher, Leitch *et al.* 1985). This finding was a landmark in understanding placental development, as it provided evidence that adhesion to a permissive extracellular matrix was sufficient to stimulate CTB proliferation and migration.

Later studies confirmed and extended these findings revealing that culture of human villi on an ECM induced differentiation along the invasive pathway, with developing CTB columns up-regulating cell-surface expression of  $\alpha 5\beta 1$  and  $\alpha 1\beta 1$ , fibronectin and down regulating  $\alpha 6\beta 4$  expression, as seen *in vivo* (Aplin, Haigh *et al.* 1999; Genbacev, Schubach *et al.* 1992). This validates the use of villous placental explants as *in vitro* models for the study of trophoblast differentiation along the invasive pathway.

### ***1.3.4 In vivo study of placentation***

Rodents have commonly been used in studies investigating placental function as they display haemochorial placentation and the advent of gene knockout and transgenic mice and rats has facilitated such studies. However, the guinea pig offers additional advantages for the study of placentation and extrapolation to humans.

#### ***1.3.4.1 Guinea pig placentation***

The guinea pig, like the human, displays an invasive, haemochorial (a single trophoblast layer in contact with maternal blood), discoidal placenta. The amniotic cavity, which is formed by cavitation, and the development of the allantoic mesenchyme are similar in both species (Pijnenborg, Robertson *et al.* 1981). In the guinea pig, trophoblasts migrate and invade arteries that traverse the placenta, promoting



maternal blood flow to it, as occurs in humans. In addition, the guinea pig placenta exerts similar endocrine control of pregnancy to that of the human in that it acquires the ability to substitute for the ovaries producing abundant progesterone and estrone, to maintain pregnancy (Enders 1965).

Guinea pig pregnancy lasts for 59 to 72 days, with a litter of two to five fetuses, which are precocious at birth. The blastocyst adheres to the uterine epithelium anti-mesometrially on day 5 or 6 post-conception. By the 7<sup>th</sup> day, the blastocyst is completely implanted in the maternal endometrium (Kaufmann 1969; Kaufmann and Davidoff 1977) (Figure 1.8a). Proliferation and differentiation of the trophoblast into a nucleated mass of STB and an underlying, thin layer of CTB enables STB invasion towards the uterine cavity (Figure 1.8b). By the 10<sup>th</sup> day, this invasion has caused the original uterine lumen to disappear, whilst a new uterine lumen begins to form anti-mesometrially. Projections of the syncytium called basal syncytial sprouts perforate the yolk sac, which surrounds the conceptus, like roots of a tree (Figure 1.8c). These syncytial sprouts proliferate intensely and invade the maternal endometrial decidua developing the junctional zone, through which maternal arteries will course into the main placenta (Kaufmann 1969; Kaufmann and Davidoff 1977).

Within the next few days, the syncytium transforms into a sponge-like area (trophospongium) as localised areas of the STB disintegrate forming lacunae (as in humans) (Figure 1.8d). Due to expansion of the STB, the basal syncytial sprouts come into contact with and erode endometrial blood vessels, allowing blood to flow into the trophospongium by the 12<sup>th</sup> day of gestation. On day 14, the extraembryonic mesoderm associated with the allantois called the central excavation, extends into the syncytium acquiring a mushroom-like shape with finger-like lamellar projections of mesenchyme lined by CTB (Figure 1.8e). The roof of this central excavation will form the main placenta-subplacenta boundary, with the main placenta proximal to the developing fetus.

From this central excavation, the extraembryonic mesenchyme proliferates and increasingly invades the trophospongium radially around the connecting stalk causing intermingling of connective tissue with the syncytium of the main placenta (Figure 1.8f). The trophospongium, interspersed with mesenchyme, forms a convoluted structure, known as the labyrinth with trophospongium septae (called the interlobium) extending in the direction of the uterus as well as towards the embryo (Kaufmann and Davidoff 1977). On day 14 of gestation, mesenchymal cells in the labyrinth begin to differentiate into fetal capillaries and from day 21 onwards, extensive capillarization occurs (Kaufmann and Davidoff 1977).

NOTE: This figure is included on page 23 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.8 Schematic representation of placental development in the guinea pig.** By the 7<sup>th</sup> day of gestation, the blastocyst is completely implanted in the maternal endometrium (**a**). Proliferation and invasion of the syncytiotrophoblast (STB) and underlying cytotrophoblast towards the primary uterine lumen (PUC) on gestational day 9, causes it to disappear, while a new uterine lumen forms (NUC) (**b**). Projections of syncytium (basal sprouts) perforate the yolk sac as seen on the 10<sup>th</sup> day of gestation (**c**). By day 12, the allantois attaches to the chorion, as the syncytium transforms into the trophospongium (TS, **d**). The extraembryonic mesoderm progressively invades the TS, forming a mushroom shape by the 15<sup>th</sup> day of gestation (**e**). The extraembryonic mesoderm invades the TS radially around the connecting stalk and differentiates into fetal capillaries (day 20-32 of gestation, **f**).  
Modified from Kaufmann and Davidoff, 1977.

Furthermore, rapid proliferation of the mesenchyme into projections allows growth of the labyrinth from the 22<sup>nd</sup> day of gestation. Within the labyrinth, modulation of the exchange interface occurs. Trophoblast encompasses maternal blood spaces on one side and fetal vessels on the other. These thin to create a reduced barrier for exchange (Enders 1965), allowing for efficient substrate and gas transfer between the two circulations.

In contrast to the labyrinth, the interlobium region of the mature main placenta is devoid of fetal capillaries. It is, however, interspersed with maternal blood spaces. Interlobial trophoblasts are thought to resorb substrates from the maternal circulation permitting synthesis of structural proteins as well as energy generation for growth of the labyrinth until term (Kaufmann and Davidoff 1977).

In the subplacenta, at around day 20, a branching network of STB forms by thickening of the lamellar folds that eventually extend centrally into the basal syncytial sprouts (Kaufmann and Davidoff 1977), similar in structure to the secondary villi in human placentation. Extraembryonic mesenchyme of the allantois differentiates into fetal blood vessels, which course through the interlobium and labyrinth and extend into the basal syncytial sprouts of the subplacenta, forming the primitive fetal circulation.

As early as 15 days of gestation, trophoblasts have been identified within the myometrium and surrounding maternal blood vessels (Carter, Tanswell *et al.* 1998) (Figure 1.9). At 35 days of gestation, the maternal myometrial and to a lesser extent, the mesometrial arteries that traverse the placenta and the myometrium, respectively, are invaded by trophoblasts (Hees, Moll *et al.* 1987; Nanaev, Chwalisz *et al.* 1995a; Verkeste, Slangen *et al.* 1998) (Roberts *et al.*, unpublished). This is associated with dramatic increases in uterine arterial dilatation, resulting in a 2 to 3-fold increase in vessel diameter (Kaufmann and Davidoff 1977). There appear to be two populations of endovascular trophoblasts within the guinea pig maternal arteries, the syncytiotrophoblast, sometimes referred to as multinucleated giant trophoblasts and the uninuclear trophoblasts that are aligned as overlapping sheets rather than a syncytium. The origin of these invasive trophoblasts and whether they perform different functions is undefined. It has been demonstrated, however, that the trophoblasts that surround blood vessels and to a lesser extent, endovascular trophoblasts, induce changes in the vasculature through the synthesis of nitric oxide, such that the vessels become widely dilated with a disorganised muscle layer that enables perfusion of blood into the placenta (Nanaev, Chwalisz *et al.* 1995a).

NOTE: This figure is included on page 25 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.9 Trophoblast invasion of the maternal vasculature in the guinea pig.**

Trophoblast (t) can be seen within the maternal spiral arteries (mv) on day 15 (A) and 35 (B) of gestation by immuno-labelling for cytokeratin (CK)  
Photomicrograph from Carter et al., 1998.

Around day 50, intra-arterial trophoblasts progressively replace the endothelium and destroy the maternal artery walls and its elastic laminae (Hees, Moll *et al.* 1987; Nanaev, Chwalisz *et al.* 1995a; Verkeste, Slangen *et al.* 1998) in a similar fashion to endovascular EVT's in the spiral arterioles in humans. In the guinea pig, trophoblast invasion of the adventitial layer also induces dedifferentiation of arterial smooth muscle cells (Nanaev, Chwalisz *et al.* 1995b; Nanaev, Kosanke *et al.* 2000a; Nanaev, Kosanke *et al.* 2000b). By term, these cells have been seen to migrate more than three quarters the way along the mesometrial arteries (Verkeste, Slangen *et al.* 1998) (Roberts *et al.*, unpublished). There is a 13-fold increase in mean cross-sectional area of these vessels (Moll, Espach *et al.* 1983), which have reduced vasculature reactivity compared to the non-pregnant state and in mid gestation (Clausen, Larsen *et al.* 2003). As gestation proceeds, the labyrinth expands at the expense of the interlobial syncytium (Kaufmann and Davidoff 1977), decreasing the thickness of the STB layer and increasing the surface area for diffusion across the STB (Roberts, Kind *et al.* 2002) (Figure 1.10).

### 1.3.5 Factors influencing the placenta

The formation of the placenta as a suitable feto-maternal interface involves spatial and temporal regulation of trophoblast proliferation, differentiation and invasion. Environmental factors including oxygen concentration, extracellular components (collagen type I, decorin, PAls, vitronectin), growth factors (insulin-like growth factor (IGF)-I and -II, epidermal growth factor, transforming growth factor (TGF)- $\alpha$  and - $\beta$ , leukaemia inhibitory factor, vascular endothelial growth factor), cytokines (colony stimulating factor, interleukin-1 $\beta$ , -6, -10 and -15, leptin) and hormones (progesterone, human gonadotropin) influence trophoblast function through autocrine and paracrine actions (Morrish, Dakour *et al.* 1998). The combination and concentration of these present at any one time regulate trophoblast function. As there is a greater body of evidence to suggest that insulin-like growth factor-II plays a critical and essential role in the formation of the placenta and may modulate nutrient partitioning between the mother and conceptus, we have primarily focused our studies on this factor. We have also discussed the effects of IGF-I on trophoblast function and pregnancy outcome for comparison with IGF-II actions. Oxygen concentration and TGF $\beta$ 1 will also be discussed as previous studies performed in our laboratory suggest an interaction of IGF-II with these factors in controlling cytotrophoblast function.

#### 1.3.5.1 Insulin-like growth factor (IGF)-axis

The insulin-like growth factors (IGFs, also known as somatomedins) belong to a family of peptides also consisting of insulin, relaxin and nerve growth factor, which exhibit highly homologous amino acid sequences. They are single chain peptides composed of A and B domains which are cross-linked by

NOTE: This figure is included on page 27 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.10 Representative illustration of the guinea mature placenta.**  
Labyrinth, interlobium and subplacenta components are indicated.  
Diagram from Kaufman and Davidoff, 1977

three disulphide bridges, with molecular weights of approximately 7.5kDa. IGFs share 50% homology with human pro-insulin and have a short D extension peptide at the carboxyl terminal of the A domain that is not present in either pro-insulin or insulin (Rinderknecht and Humbel 1978; Sara and Hall 1990). Two different isoforms of IGF have been identified, IGF-I and IGF-II that share 62% similarity with each other (D'Ercole 1996; Rinderknecht and Humbel 1978) and have been described to possess insulin synergizing activity (Mauras and Haymond 2005).

IGFs have metabolic, mitogenic, anti-apoptotic, differentiating and transformation effects on a wide variety of cell types (Jones and Clemmons 1995). The diversity of IGF functions is achieved through binding to receptors with different affinities and different effects. IGFs can bind to two types of IGF receptor, IGF1R and IGF2R, as well as the insulin receptor (InsR), and IGF1R-InsR hybrids. IGFs can bind to six IGF binding proteins (IGFBP-1 to IGFBP-6), which in turn are regulated by IGFBP proteases and several other IGFBP-interacting molecules and -related peptides (Figure 1.11). This complex IGF system regulates and propagates IGF actions in several tissues.

#### 1.3.5.1.1 IGF-I

The human IGF-I gene is located on chromosome 12q22 (Rotwein 1991) and consists of 6 exons and 5 introns (LeRoith and Roberts 1993). The IGF-I gene contains different promoters and multiple transcription start sites, which allow the generation of multiple IGF-I mRNA transcripts (LeRoith and Roberts 1993). IGF-I is a key growth factor in many reproductive processes including, folliculogenesis, oocyte maturation, sperm motility and fertilisation, uterine proliferation and pre-implantation embryo and fetal development (as reviewed by (Kabir-Salmani, Shiokawa *et al.* 2003)).

#### 1.3.5.1.2 IGF-II

The human IGF-II gene spans 30kb of chromosomal DNA and is located on chromosome 11p15 (de Pagter-Holthuisen, Hoppener *et al.* 1985). It consists of nine exons, of which exons 7, 8 and the first part of exon 9 encode IGF-II. Also four promoters are active in a tissue-specific fashion during growth and development. IGF-II over-expression is implicated in a variety of human malignancies, and therefore IGF-II is thought to play a role in tumourgenesis (as reviewed by (Moschos and Mantzoros 2002)). There is also evidence of IGF-II over-expression in patients with Beckwith-Wiedemann overgrowth syndrome (Morison, Becroft *et al.* 1996). Within the fetus and the placenta, the majority of transcripts are regulated by the third promoter (Daimon, Johnson *et al.* 1992) however, in mice, the *Igf2* P0 transcript has been identified and is responsible for trophoblast expression of IGF-II (Constancia, Hemberger *et al.* 2002)

NOTE: This figure is included on page 29 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.11 Figure depicting the insulin-like growth factor (IGF) system.**  
IGF-I and IGF-II must be cleaved from insulin-like growth factor binding proteins (IGFBPs) so that they may interact with the type 1 or 2 IGF receptors (IGF1R and IGF2R) or insulin receptor (InsR). ALS = acid liable subunit.  
Diagram from Gicquel, 2006



#### 1.3.5.1.3 Regulation of IGF abundance

The IGF system is heavily influenced by nutrition, pregnancy, cortisol, sex steroids (particularly estrogen), growth hormone (except in guinea pigs) and oxygen concentration and to a lesser extent by other molecules such as ghrelin and leptin. Additionally, during pregnancy, considerable changes in the maternal IGF system occur, which are thought to be important in determining the partitioning of nutrients between the mother and the fetus and regulation of conceptus growth.

#### 1.3.5.1.4 IGF expression

IGFs are expressed in most tissues, the liver being a major source and their highest concentrations are found circulating in the blood, except in rats and mice where IGF-II is undetectable in the circulation postnatally. Both *Igf* genes are expressed in the embryo and then the fetal tissues from the earliest stage of pre-implantation development to the final phase of tissue maturation just before birth (Fowden, Li *et al.* 1998; Hill, Petrik *et al.* 1998; Watson, Watson *et al.* 1994). During mid to late gestation, *Igf2* gene expression is widespread in fetal tissues and is more abundant than *Igf1* gene expression in rodents, ruminants and humans (Delhanty and Han 1993; Gicquel and Le Bouc 2006; Hill 1990). The concentration of IGF-II in the fetal circulation is also much higher than IGF-I, with a three to ten-fold difference in all species studied (Fowden 2003). In rats and mice, IGF-II expression disappears from most tissues except the brain by weaning, with the consequence that IGF-II is virtually undetectable in adult plasma (Lee, Pintar *et al.* 1990; Singh, Rall *et al.* 1991). In humans and other species, like the guinea pig, IGF-II remains the most abundant IGF in the circulation throughout life (D'Ercole 1996; Han, Lund *et al.* 1988; Holly 1998).

#### 1.3.5.1.4.1 IGFs in the mother during pregnancy

During normal pregnancy, substantial changes in the maternal IGF system occur, suggesting that endocrine IGFs may play a dominant role in the regulation of placental and fetal growth. Maternal plasma concentrations of IGF-I increase early in most species (Donovan, Giudice *et al.* 1991; Hills, English *et al.* 1996; Nason, Binder *et al.* 1996; Sohlstrom, Fernberg *et al.* 2001; Sohlstrom, Katsman *et al.* 1998b; Travers, Madon *et al.* 1990; Wallace, Da Silva *et al.* 1997; Wilson, Bennett *et al.* 1982), whilst the abundance of circulating IGF-II increases in some species (Nason, Binder *et al.* 1996), including humans (Gargosky, Moyses *et al.* 1990; Wilson, Bennett *et al.* 1982), but remains unchanged in others (Donovan, Giudice *et al.* 1991; Sohlstrom, Katsman *et al.* 1998b). Nonetheless, IGF-II is the most abundant IGF in the maternal circulation during pregnancy in the majority of species.

Non-hepatic tissues are thought to contribute substantially to maternal circulating IGF pools during pregnancy. Studies in guinea pigs have demonstrated that *Igf1* gene expression is induced in maternal adipose tissue by pregnancy and that the main source of IGF-II during pregnancy is the placenta (Olausson and Sohlstrom 2003). Pregnancy also alters the abundance and stability of the IGFBPs in the maternal circulation, which in turn may influence IGF bioavailability. For instance, in the guinea pig, IGFBP-1 and IGFBP-2 concentrations increase in the circulation in late pregnancy, whilst that of IGFBP-4 increases in early gestation and drops to non-pregnant concentrations later (Sohlstrom, Katsman *et al.* 1998b).

While maternal IGFs do not cross the placenta in physiologically important quantities (Brown and Thorburn 1989), they may act on the placenta and maternal tissues to regulate nutrient allocation between the mother, placenta and fetus (Wallace, Da Silva *et al.* 1997). IGFs may directly or indirectly influence maternal physiological adaptation to pregnancy.

#### *1.3.5.1.4.2 IGFs at the feto-maternal interface*

Both IGFs are expressed at the feto-maternal interface during pregnancy, with similarities in cellular localization across species. In general, IGF-II is more abundantly expressed by the placenta (Dhara, Lalitkumar *et al.* 2001; Fowden 2003; Han and Carter 2000) than IGF-I and is a more potent regulator of trophoblast function (Hamilton, Lysiak *et al.* 1998).

##### *1.3.5.1.4.2.1 IGF-I expression at feto-maternal interface*

IGF-I is expressed in the human and other species endometrium across the menstrual cycle, primarily in mid to late proliferative and early secretory phases (as reviewed by (Giudice, Mark *et al.* 1998; Han and Carter 2000; Nayak and Giudice 2003)), and is thought to be a mediator of the mitotic actions of estradiol (Sato, Wang *et al.* 2002). During pregnancy, IGF-I is thought to play a role in decidualisation because it is predominantly expressed by decidual stromal cells (Correia-da-Silva, Bell *et al.* 1999; Dhara, Lalitkumar *et al.* 2001; Han and Carter 2000). In the human placenta, the main source of IGF-I in the first trimester is the villous mesenchyme (Lacey, Haigh *et al.* 2002). In the guinea pig, IGF-I is expressed weakly in the main placental labyrinth syncytium (compared to IGF-II) and in the subplacental mesenchyme and CTB (Han, Carter *et al.* 1999). These findings suggest a paracrine influence of IGF-I on trophoblast function.

#### 1.3.5.1.4.2.2 IGF-II expression at the feto-maternal interface

In situ hybridisation studies have revealed that, in the human placenta, *Igf2* is exclusively expressed by CTBs as early as 6 weeks of gestation, with highest levels expressed by the invading EVT in the uterine tissue and vasculature (Han, Bassett *et al.* 1996; Ohlsson, Holmgren *et al.* 1989; Ohlsson, Larsson *et al.* 1989) (Figure 1.4). The mesenchyme and endothelium, however, also express *Igf2*, but at much lower levels compared with CTB (Ohlsson, Larsson *et al.* 1989). *Igf2* mRNA is absent in the villous STB (Han, Bassett *et al.* 1996).

This pattern of *Igf2* expression by trophoblast at the invading front occurs across species. For instance, IGF-II is abundantly synthesized by the invasive multinucleate and uninucleate trophoblasts around and within the maternal arterioles in guinea pigs (Han, Carter *et al.* 1999), EVT in the rhesus monkey, the invasive trophoblast glycogen cells in the mouse (Redline, Chernicky *et al.* 1993) and all trophoblast populations in the rat (Zhou and Bondy 1992). These ontogenic studies suggest that IGF-II is important in trophoblast invasion and this function is conserved between species.

#### 1.3.5.1.5 IGF receptors

IGFs can bind to two types of IGF receptor; IGF1R and IGF2R, as well as the InsR, all with differing affinity. IGF1R, structurally related to the InsR, is a disulfide-linked heterotetrameric ( $\alpha_2\beta_2$ ) transmembrane glycoprotein with an extracellular ligand-binding  $\alpha$ -subunit and intracellular  $\beta$ -subunit with tyrosine kinase activity (Nissley, Haskell *et al.* 1985). IGF1R binds IGF-I with a much higher affinity than IGF-II, and insulin with little or no affinity (Germain-Lee, Janicot *et al.* 1992). In contrast, IGF2R is a single transmembrane receptor, which is identical to the cation-independent mannose-6-phosphate (M6P) receptor consisting of a large extracellular domain (15 contiguous repeats) and a small cytoplasmic tail that lacks tyrosine kinase activity (Ludwig, Le Borgne *et al.* 1995). IGF2R possesses an IGF-II binding site in the extracellular domain, distinct from two M6P-binding sites to which IGF-II binds with 700-fold higher affinity than IGF1R. Conversely, IGF-I binds to IGF2R with 500-fold less affinity than IGF-II, while insulin binds IGF2R with little or no affinity (Jones and Clemmons 1995).

While the affinity of insulin binding to IGF1R is 1/500 to 1/1000 than that of the IGFs, IGFs can act through the InsR. IGF-II can bind to the InsR with an affinity that is 1/10 that of insulin and 5-10 times greater than that of IGF-I (De Meyts, Wallach *et al.* 1994). In addition, there are insulin-IGF hybrid receptors, composed of an  $\alpha\beta$  heterodimer of the InsR and an  $\alpha\beta$  heterodimer of the IGF1R, to which the IGFs can bind with high affinity and insulin with 10-fold lower affinity (Soos, Field *et al.* 1993).

Human trophoblasts express both IGF receptors, InsR (Abu-Amero, Ali *et al.* 1998; Desoye, Hartmann *et al.* 1997; Holmes, Porter *et al.* 1999; Jones, Hartmann *et al.* 1993; Milio, Hu *et al.* 1994; Ohlsson, Holmgren *et al.* 1989) and IGF1R-InsR hybrids (Soos, Field *et al.* 1993) providing support for an IGF-II-mediated autocrine and/or paracrine control of placental development. Further studies are required to elucidate which receptor (IGF1R, IGF2R, InsR or receptor hybrids) mediates IGF-I and IGF-II effects on placentation.

#### 1.3.5.1.5.1 IGF1R

Traditionally, the biological activities of IGF-I and IGF-II were primarily thought to be mediated through the IGF1R. A variety of studies have shown that interaction between IGF-I or IGF-II with IGF1R leads to cell proliferation in numerous cell types, which involves activation of the insulin receptor substrate-1-dependent intracellular signalling pathways (Jones and Clemmons 1995). More recently however, there is evidence that IGF1R phosphorylates FAK and paxillin which lead to cytoskeletal remodelling and migration of cells *in vitro* (BurrIDGE, Turner *et al.* 1992; Casamassima and Rozengurt 1998). This has been demonstrated in human EVT cells, where IGF-I binding of IGF1R activated  $\alpha v\beta 3$  (Kabir-Salmani, Shiokawa *et al.* 2003) and  $\alpha 5\beta 1$  (Clemmons and Maile 2003) integrins. Consistent with this, IGF1R number in trophoblast membranes isolated from term human placenta has been positively correlated with placental and birth weights (Diaz, Cardenas *et al.* 2005).

#### 1.3.5.1.5.2 IGF2R

Rather than having a role in signal transduction, IGF2R initially appeared to function only as a clearance receptor for IGF-II, as mice deficient in the *Igf2r* gene have increased serum and tissue levels of IGF-II (Ludwig, Eggenschwiler *et al.* 1996). This receptor has a recognised function in the transport of various ligands through the endosomal pathway. It has been reported that only about 10% of the cell's IGF2R is present on the cell surface whilst the remaining 90% is found intracellularly on either the Golgi apparatus or the endosomal and lysosomal compartments (Boker, von Figura *et al.* 1997). IGF2R is thought to control the extracellular IGF-II concentration by mediating the endocytosis of IGF-II and its subsequent degradation in lysosomes (Jones and Clemmons 1995). IGF2R has been described as a tumor-suppressor, as its downregulation caused an accumulation of IGF-II that stimulated growth upon binding to IGF-R1 and InsR in choriocarcinoma cell lines (Lee, Rushlow *et al.* 2001).

The use of the IGF-II analogue, [Leu<sup>27</sup>]-IGF-II (Leu<sup>27</sup>-IGF-II), which cannot bind to the IGF1R nor the InsR, has provided evidence that IGF2R activity does not alter DNA synthesis (Beukers, Oh *et al.* 1991)

but influences myoblast cell differentiation (Rosenthal, Hsiao *et al.* 1994) and rhabdomyosarcoma (Minniti, Kohn *et al.* 1992) and endothelial cell migration (Volpert, Jackson *et al.* 1996). The cytoplasmic tail of IGF2R contains a putative inhibitory G protein (Gi) coupled receptor binding site (Murayama, Okamoto *et al.* 1990), which is required for IGF-II-IGF2R dependent signalling (Ikezu, Okamoto *et al.* 1995). Furthermore, in mice, interaction of the placental angiogenic factor, proliferin with the IGF2R, supports a role for the receptor in mediating endothelial cell migration via this intracellular signalling pathway (Groskopf, Syu *et al.* 1997). Consistent with this, hCG stimulation of migration and invasion of the choriocarcinoma cell line, JEG-3, has been shown to involve IGF2R (Zygmunt, McKinnon *et al.* 2005).

#### 1.3.5.1.5.3 *InsR*

Traditionally, InsR was thought to solely mediate metabolic actions within cells. Within the last decade however, studies have suggested that IGFs can interact with InsR and may be involved in their growth-promoting effects (Frasca, Pandini *et al.* 1999). Studies performed in IGF1R deficient cells over-expressing the InsR demonstrated that IGF-II (but not IGF-I) can stimulate cell proliferation through InsR and that this stimulation was at least 2-fold higher than insulin (Morrione, Valentinis *et al.* 1997). This effect is postulated to have occurred through the alternatively spliced form of the InsR, InsR-A, which binds with high affinity to IGF-II, but not IGF-I (Denley, Brierley *et al.* 2006). Indeed, IGF-II can signal through InsR-A and induce cancer cell proliferation, invasion and protection from apoptosis (Sciacca, Mineo *et al.* 2002). Interestingly, placental InsR affinity (Kd values) assessed by binding assays was reduced in trophoblast membranes isolated from term placenta obtained from preeclamptic women compared to normotensive women (Diaz, Cardenas *et al.* 2005). These studies suggest that InsR, particularly the InsR-A may play a role in placental development and pregnancy outcome.

#### 1.3.5.1.5.4 *IGF1R-InsR hybrids*

IGF1R-InsR hybrids represent the major form of IGF1R in human term placenta, with immunoblotting and immunoprecipitation studies revealing that 70% of IGF1R in the placenta is heterodimerised with InsR (Baillyes, Nave *et al.* 1997; Soos and Siddle 1989). Heterodimers of InsR and IGF1R display lower affinity for insulin than InsR isoforms alone (Pandini, Frasca *et al.* 2002), therefore their formation may be a mechanism for switching the major ligand binding InsR from insulin to IGFs (Baillyes, Nave *et al.* 1997).

Given the major effects of IGFs on trophoblast function, the relative abundance of hybrid receptors in the placenta and their involvement in mediating IGF effects on cell function, these receptors may play a

dominant role orchestrating placental development. Further studies are required to elucidate this possibility.

#### 1.3.5.1.6 *In vivo studies of the IGFs*

##### 1.3.5.1.6.1 *Rodent studies*

Direct support for a role of both IGFs in fetal growth and for IGF-II, but not IGF-I, in placental development has been provided by the generation of targeted gene deletion and transgenic mice (Table 1.1). Deletion of either *Igf* gene results in a similar degree of fetal growth restriction, where birth weight is 60% of normal (Baker, Liu *et al.* 1993; DeChiara, Efstratiadis *et al.* 1990).

In mice, deficiency of a functional *Igf2* gene reduces placental weight by 17% on day 13.5 and by 25% on day 16.5 of gestation, with a fetal weight reduction of 40% at this time (term = 19 days) (Baker, Liu *et al.* 1993; DeChiara, Efstratiadis *et al.* 1990). There is a 50% reduction in the average number of cells in the labyrinth and junctional zones in the *Igf2* deficient placenta in late gestation. The latter contains a smaller proportion of invasive giant cells and glycogen cells (Lopez, Dikkes *et al.* 1996). Glycogen cells have been thought to be analogous to human EVT and are the main source of IGF-II in late murine pregnancy (Redline, Chernicky *et al.* 1993). In contrast, in mouse chimeras with paternal duplication of chromosome 7 which results in *Igf2* over-expression (Ferguson-Smith, Cattnach *et al.* 1991) or in mice that are deficient in *Igf2r* and display elevated serum and tissue concentrations of IGF-II (Lau, Stewart *et al.* 1994; Ludwig, Eggenschwiler *et al.* 1996), placental and fetal growth is increased. This is thought to be due to the over-stimulation of IGF1R by excess IGF-II (Efstratiadis 1998). Combined, these suggest that IGF-II plays a dominant role in regulating the number and differentiation of trophoblast populations in the placenta (Fowden 2003).

IGF-II effects on fetal growth may be secondary to impacts on the placenta as placental amino acid transporter expression is altered in *Igf2* deficient mice (Matthews, Beveridge *et al.* 1999). Certainly, recombination experiments in mouse blastocysts where the inner cell mass of an *Igf2* deficient murine blastocyst was replaced with that of a wildtype are also consistent, as placental and fetal weights were reduced on day 16.5 (Gardner, Squire *et al.* 1999). Furthermore, dexamethasone administration to pregnant rats during the last third of pregnancy reduces placental expression of IGF-II and is associated with a 50% decrease in placental mass and IUGR (Ain, Canham *et al.* 2005). Additionally, deletion of the *P0* transcript in mice, which is responsible for placental-specific expression of *Igf2*, also resulted in placental and fetal growth restriction (Constancia, Hemberger *et al.* 2002). The reduced fetal growth seen in these pregnancies is thought to be due to altered placental structural differentiation, which

**Table 1.1 Fetal and placental phenotypes of mice genetically deficient in IGF or IGF receptors\***

Gene	Effect	Placenta	Fetal Weight (% WT)
<i>Igf1</i>	No plasma or tissue IGF-I	Weight = 100% WT Structure not assessed	60
<i>Igf2</i>	No plasma or tissue IGF-II	Weight = 75% WT ↓ labyrinth ↓ junctional zone ↓ glycogen and giant cells	60
<i>Igf2 P0</i>	Reduced placental IGF-II	Weight = 65% WT ↓ in all placental layers ↓ surface area for exchange ↑ thickness of exchange barrier	75
<i>Igf1r</i>	No action of IGF at IGF1R	Weight = 100% WT Structure not assessed	45
<i>Igf2r</i>	Elevated plasma and tissue IGF-II	Weight = 140% WT Structure not assessed	140

WT = wild type. \* Modified from Fowden, 2003. Refer to text for references

resulted in decreased passive permeability (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004) (Table 1.1). Furthermore, glucose transport was increased throughout and active amino acid uptake was initially up-regulated, but ultimately failed (Constancia, Angiolini *et al.* 2005). The reduction in fetal weight was comparable to that induced by global *Igf2* gene ablation, indicating that in mice, local IGF-II production is required for placental development and subsequent fetal growth.

Ablation of the *Igf1* gene also resulted in a similar reduction of birth weight to that seen with *Igf2* gene ablation, but did not affect placental weight in late gestation. IGF-I administration to pregnant rats, or increased endogenous expression in pregnant mice, increases the weight of the fetus but not that of the placenta (Gluckman and Harding 1992). A null mutation for the *Igf1r* gene also resulted in slowing of prenatal fetal growth, which is more severe than deficiency of either *Igf* (45% of normal body weight) without any effect on placental size (Baker, Liu *et al.* 1993). Furthermore, placental growth is normal in double-mutants lacking both *Insr* and *Igf1r* (Efstratiadis 1998). Although neither of these genotypes affect placenta weight, it is possible that IGF-I, IGF1R and InsR are involved in regulating placental differentiation and functional capacity thereby affecting fetal growth. However, this possibility is yet to be explored *in vivo*.

The fetus is also thought to signal to the placenta to coordinate growth and function in mice. In chimeric embryos with normal IGF-II gene expression in the placenta but IGF-II deficiency in the fetus, placental weight is reduced by 14% (Gardner, Squire *et al.* 1999). Furthermore, recent experiments comparing nutrient transfer in *Igf2 P0* and complete *Igf2* null mice, suggested that IGF-II in the fetus of *Igf2 P0* mothers signals to the placenta to up-regulate nutrient transporter expression (Constancia, Angiolini *et al.* 2005).

#### 1.3.5.1.6.2 Guinea pig studies

More information on the regulation of fetal growth by IGFs has come from studies performed on guinea pigs, which, like humans, display postnatal IGF-II in their circulation (Sohlstrom, Katsman *et al.* 1998b). In the guinea pig, major structural determinants of placental function are strongly predicted by maternal IGF-II concentration in mid pregnancy and by maternal IGF-I in late pregnancy (Roberts, Kind *et al.* 2002; Roberts, Sohlstrom *et al.* 2001b). Furthermore, in this species, food restriction alters the expression of IGF binding proteins in the maternal plasma and reduces IGF concentrations (Sohlstrom, Katsman *et al.* 1998b) through direct impacts on maternal hepatic gene expression (Grant, Kind *et al.* 2005). These alterations in the IGF system of the mother correlate with delayed structural and functional maturation of the placenta and with reduced fetal growth (Roberts, Kind *et al.* 2002; Roberts, Sohlstrom



*et al.* 2001a; Roberts, Sohlstrom *et al.* 2001b). Consistent with this, treatment of the guinea pig mother with IGF-I or IGF-II in early to mid pregnancy increases placental and fetal weights by mid gestation (Sohlstrom, Fernberg *et al.* 2001). Whether these anabolic effects of maternal exposure to elevated IGFs in early to mid gestation on the fetus and placenta are sustained to term is presently unknown. Furthermore, impacts of elevated maternal IGFs on placental development, transport and nutrient partitioning within the mother remain to be determined.

#### 1.3.5.1.6.3 Human studies

In women, reduced plasma IGF-I is associated with placental dysfunction (as indicated by altered Doppler velocimetry in umbilical arteries) and small for gestational age (Holmes, Holly *et al.* 1998; Larsen, Main *et al.* 1996; Stefanidis, Solomou *et al.* 1998) or growth restricted infants (Holmes, Montemagno *et al.* 1997). In preeclamptic pregnancies, maternal serum IGF-I concentrations at term are lower compared with those in normal pregnant women (Giudice, Martina *et al.* 1997; Halhali, Tovar *et al.* 2000) and in a separate study, maternal IGF-I concentrations in late pregnancy, correlated with placental mass (and neonatal fat mass), near term (Clapp, Schmidt *et al.* 2004). More recently, abnormalities in the post-translational processing of IGF-II from inactive pro-IGF-II into the mature peptide by proprotein convertase-4 in women has been associated with IUGR (Qiu, Basak *et al.* 2005). Altogether, these studies suggest an important role of maternal endocrine IGFs in determining pregnancy success.

Studies associating placental expression of IGF system components with pregnancy outcome have been inconsistent and inconclusive. Some studies reported increased *Igf1*, *Igf2* (Abu-Amero, Ali *et al.* 1998; Sheikh, Satoskar *et al.* 2001) and *Igf1r* (Abu-Amero, Ali *et al.* 1998) gene expression in term human placenta of IUGR pregnancies. Other studies have either shown no difference in IGF1R (Holmes, Porter *et al.* 1999) or reduced IGF1R protein expression (Laviola, Perrini *et al.* 2005) in term human placenta of growth restricted fetuses compared with normal. Another study reported that placental expression of IGF1R was comparable and InsR Kd values were higher (without an alteration in receptor abundance), in preeclamptic women compared to normal (Diaz, Cardenas *et al.* 2005). Inconsistencies between studies may relate to differences in sampling of the placenta or implantation site, clinical classification of pregnancies in which the neonate is growth restricted versus one that is small for gestational age and whether detecting gene or protein levels. Caution is warranted when drawing conclusions from observations at term as they may not necessarily reflect what is going on in early pregnancy, when placental development is most extensive and critical for optimal function.

#### 1.3.5.1.7 *In vitro studies*

##### 1.3.5.1.7.1 *IGF-I effects on placenta*

Exogenous IGF-I stimulates proliferation and secretion of hCG and hPL in first trimester villous explants (Maruo, Murata *et al.* 1995; Murata, Maruo *et al.* 1994) and choriocarcinoma cell lines (Mandl, Haas *et al.* 2002). IGF-I stimulates the proliferation and migration of mouse ectoplacental cone cells (trophoblast stem cells) *in vitro* (Kanai-Azuma, Kanai *et al.* 1993). Placental fibroblast-derived IGF-I promotes trophoblast migration in first trimester human placental villous explants (Lacey, Haigh *et al.* 2002). This is consistent with elevated MMP-9 activity following IGF-I treatment of first trimester trophoblast primary cell cultures (Hills, Elder *et al.* 2004). IGF-I also promotes trophoblast adhesion to fibronectin (Liu, Zhang *et al.* 2005) and subsequent IGF1R-dependent EVT migration through activation of  $\alpha v \beta 3$ -integrin (Kabir-Salmani, Shiokawa *et al.* 2003) and  $\alpha 5 \beta 1$  (Clemmons and Maile 2003; Kabir-Salmani, Shiokawa *et al.* 2004), assembly of focal adhesions and alterations in cytoskeletal organisation (Kabir-Salmani, Shiokawa *et al.* 2002). Consistent with this, IGF1R number in trophoblast membranes isolated from term human placenta has been positively correlated with placental and birth weights (Diaz, Cardenas *et al.* 2005).

IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (Bloxam, Bax *et al.* 1994; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998). In late gestation, IGF-I is thought to act as a survival factor for STB, as it inhibits TNF $\alpha$ - and IFN $\gamma$ -induced apoptosis in syncytialised trophoblast cultures from third trimester placenta (Smith, Francis *et al.* 2002). Additionally, in late pregnancy, IGF-I may modulate placental blood flow, as exposure to IGF-I inhibits release of vasoconstrictors, such as thromboxane B<sub>2</sub> and prostaglandin F<sub>2</sub> $\alpha$ , in human term placental explants (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995) and can stimulate the production of nitric oxide in vascular endothelial cells (Zeng and Quon 1996).

##### 1.3.5.1.7.2 *IGF-II effects on the placenta*

IGF-II stimulates glucose and amino acid uptake in cultured human CTBs (Kniss, Shubert *et al.* 1994). IGF-II stimulates DNA synthesis (Ohlsson, Holmgren *et al.* 1989) and increases MMP-2 and MMP-9 activity (Hills, Elder *et al.* 2004) in first trimester CTB cultures. Furthermore, IGF-II has been reported to stimulate EVT cell migration (Irving and Lala 1995; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001) and invasion independent of IGF1R *in vitro* (Hamilton, Lysiak *et al.* 1998) via a Gi-coupled receptor (Ikezu, Okamoto *et al.* 1995; Murayama, Okamoto *et al.* 1990) and phosphorylation of the mitogen activated protein kinases; ERK-1 and ERK-2 (Hamilton, Lysiak *et al.* 1998). IGF-II also

promotes murine trophoblast differentiation, by transforming ectoplacental cone cells (trophoblast stem cells) into trophoblast giant cells *in vitro* (Kanai-Azuma, Kanai et al. 1993).

IGF-II may also promote placental angiogenesis during pregnancy. It has been shown that IGF-II, secreted by human hepatocellular carcinoma cells, promotes vasculogenesis in a three-dimensional angiogenesis assay *in vitro* as well as in a chicken chorioallantoic membrane assay *in vivo* (Bae, Lee et al. 1998). Indeed, IGF-II induces the expression of vascular endothelial growth factor (VEGF) in human hepatoma and keratinocyte cells (Kim, Bae et al. 1998; Kwon, Kwon et al. 2004). It has also been reported that IGF-II acts through IGF2R to promote placental angiogenesis and vascular remodelling (Herr, Liang et al. 2003). IGF-II may also promote angiogenesis through synergising with epidermal growth factor (Lee, Bae et al. 2004) or indirectly through activating the hypoxia response pathway and/or promote adaptation to hypoxia, as it increases the expression of hypoxia inducible factor (HIF)-1 $\alpha$  protein and induces *Vegf* transcription (Feldser, Agani et al. 1999).

#### 1.3.5.1.8 *Insulin-Like Growth Factor Binding Proteins (IGFBPs)*

The bioavailability of IGFs, but not insulin, is determined at a cellular level by six specific IGFBPs that have equal or higher affinity for IGFs than the IGF receptors. In general, IGFBPs have a greater affinity for IGF-II than IGF-I, however, binding capacity and effects of IGFBPs on cell function may be affected by IGFBP phosphorylation (Yu, Iwashita et al. 1998) or glycosylation (Hossenlopp, Segovia et al. 1990). The six high-affinity IGFBPs share 50% homology with each other and up to 80% homology with the corresponding IGFBP from different mammalian species (Kelley, Oh et al. 1996; Rechler 1993). The main source of IGFBPs in the circulation is the liver. Each IGFBP has a unique tissue and developmental stage-specific pattern of expression, suggesting that they have different, regulated functions during development and tissue processes. Depending on the IGFBP, it may be regulated by a number of factors, including nutrition, insulin, glucagon, glucocorticoids, cytokines, hormones, hypoxia, growth hormone, vitamin D and retinoic acid.

##### 1.3.5.1.8.1 *IGFBP functions*

IGFBPs have several functions, including transporting and prolonging the half-life of IGFs in the circulation, such that less than 1% of the IGFs in the circulation are present in the free form, which is mainly due to the high abundance of plasma IGFBP-3, which on its own, is responsible for binding 75-80% of the IGFs in the circulation (Jones and Clemmons 1995). IGFBPs regulate the transport of IGFs between the intra- and extravascular spaces. For instance, IGFBP-3 and IGFBP-5 circulate as large ternary complexes, also consisting of an acid-labile subunit (also synthesised by the liver) and IGF

(Coverley and Baxter 1997), which must be shed in order for IGFs to leave the circulation. IGFBPs are also thought to localise IGFs to specific cell types and modulate both IGF binding to receptors and growth promoting actions (Clemmons 1993; Clemmons 1998; Clemmons, Busby *et al.* 1995). Therefore, IGFBPs are considered to regulate the endocrine actions of IGFs.

In addition, IGFBPs contain functional domains which enable IGF-independent actions (Figure 1.12). For instance, IGFBPs may inhibit or enhance cell growth and induce of apoptosis (Wetterau, Moore *et al.* 1999). These functions have best been described for IGFBP-3 and IGFBP-5, which are known to interact with the cell surface expressed type V transforming growth factor- $\beta$  receptor (T $\beta$ R-V) (Baxter 2000; Huang, Ling *et al.* 2003; Leal, Huang *et al.* 1999). Furthermore, IGFBP-3 binds and activates intracellular signalling via the transforming growth factor receptor type-II and -I heteromeric complex and inhibits breast cancer growth (Fanayan, Firth *et al.* 2002; Fanayan, Firth *et al.* 2000; Oh, Muller *et al.* 1993) and smooth muscle cell proliferation (Kuemmerle, Murthy *et al.* 2004). Additionally, IGFBP-3 and IGFBP-5 can translocate to the nucleus (Schedlich, Le Page *et al.* 2000), supporting the concept that IGFBPs have functions unrelated to direct IGF actions.

Furthermore, studies have provided evidence that IGFBPs can interact with extracellular matrix proteins via heparin-binding domains (Figure 1.13) (Arai, Busby *et al.* 1996; Arai, Parker *et al.* 1994; Russo, Bach *et al.* 1997; Russo, Schutt *et al.* 2005) and modulate integrin-induced cell proliferation, migration and invasion through the expression of an Arg-Gly-Asp (RGD) motif (Chakraborty, Gleeson *et al.* 2002; Irwin and Giudice 1998; Russo, Schutt *et al.* 2005; Schutt, Langkamp *et al.* 2004; Wang, Hu *et al.* 2006), both independently of IGF actions (Gleeson, Chakraborty *et al.* 2001; Irwin and Giudice 1998) and by enhancing IGF-II actions *in vitro* (Hamilton, Lysiak *et al.* 1998). Due to functional redundancy between IGFBPs, identifying and defining the specific effects of each IGFBP has been difficult.

#### 1.3.5.1.8.2 Pregnancy and IGFBPs

At the fetomaternal interface, IGFBP-1 to IGFBP-6 are predominantly expressed by the decidua during human pregnancy, with IGFBP-1 being the most abundant (Hill, Clemmons *et al.* 1993). Additionally, IGFBP-3, IGFBP-4 and IGFBP-5 are also expressed by the chorionic mesoderm (Han and Carter 2000). Given IGFBP expression at the fetomaternal interface, IGFBPs may play a significant role in modulating IGF actions in the placenta, as well having IGF-independent effects.

In the maternal circulation, the abundance and stability of the IGFBPs change during pregnancy (Donovan, Giudice *et al.* 1991; Hills, English *et al.* 1996; Monaghan, Godber *et al.* 2004). This normally

NOTE: This figure is included on page 42 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.12 Figure depicting the generalised structure of an insulin-like growth factor binding protein (IGFBP) and functional domains.**

ALS = acid labile subunit, RGD = Arg-Gly-Asp motif.

Diagram from Firth and Baxter 2002

occurs as a result of post-translational modification and protease activity. Proteases for which IGFBPs are substrates include plasmin, serine proteases, matrix metalloproteinases, tissue plasminogen activator, thrombin, prostate-specific antigen and pregnancy-associated plasma protein-A (PAPP-A) (Gargosky, Pham *et al.* 1992; Wetterau, Moore *et al.* 1999). Indeed, proteolysis is thought to account for the virtual absence of intact IGFBP-2, IGFBP-3, and IGFBP-4 in the peripheral circulation of the mother during pregnancy (Irwin, Suen *et al.* 2000).

Alterations in the abundance of IGFBPs in the maternal circulation have the potential to affect fetal growth, through indirectly altering the bioavailability of IGFs required for essential placental growth and function for fetal development. For instance, maternal circulating IGFBP-1 is negatively correlated with birth and placental weight (Hills, English *et al.* 1996) and increased concentrations near term are associated with severe pre-eclampsia (Anim-Nyame, Hills *et al.* 2000; Wang, Lee *et al.* 1996) and intrauterine growth restriction caused by uteroplacental insufficiency (Langford, Blum *et al.* 1994). Furthermore, IGFBP-1 over-expression in mice results in IUGR (Murphy 2000; Murphy, Rajkumar *et al.* 1995), which is probably linked to perturbations in placental development by decidual over-expression of IGFBP-1, and thus reduced IGF-II actions at the feto-maternal interface, leading to placental insufficiency (Crossey, Pillai *et al.* 2002). Impaired proteolysis of IGFBPs may be an additional mechanism for altering the bioavailability of IGFs during pregnancy. For example, reduced maternal circulating PAPP-A concentrations, the IGFBP-4 specific protease in early pregnancy have been associated with an increased risk for IUGR (Smith, Stenhouse *et al.* 2002) and miscarriage (Tong, Marjono *et al.* 2004).

### 1.3.5.2 Hypoxia

Hypoxia is known to influence the expression of IGF system components and therefore may play a role in the regulation of the IGF system at the fetal-maternal interface during pregnancy.

During early pregnancy, placentation occurs in a physiological but hypoxic environment, as the extravillous trophoblasts invading uterine spiral arteries initially form endovascular plugs which occlude them and prevent maternal blood from entering the IVS (Burton, Jauniaux *et al.* 1999; Hamilton and Boyd 1960; Harris and Ramsey 1966; Hustin and Schaaps 1987). Direct measurements of oxygen tension between 8 to 10 weeks of gestation have shown that the partial pressure of oxygen in the placenta (<20mmHg) is significantly lower than in the endometrium (~40mmHg) (Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992). By 12 to 13 weeks of gestation, oxygen tension in the IVS increases nearly 3-fold, to >60mmHg, which is not significantly different from the endometrium (Burton, Jauniaux

*et al.* 1999; Rodesch, Simon *et al.* 1992), and is associated with changes in antioxidant enzymes (Jauniaux, Watson *et al.* 2000). This indicates that CTBs have begun to substantially remodel the spiral arteries and maternal blood is flowing into the IVS by this time (Jaffe and Woods 1993; Jauniaux, Jurkovic *et al.* 1992; Jauniaux, Watson *et al.* 2000). Certainly, endovascular trophoblastic plugs progressively loosen from ~10 weeks of gestation, exposing the developing placenta to maternal blood flow (Burton, Jauniaux *et al.* 1999; Jauniaux, Greenwold *et al.* 2003; Rodesch, Simon *et al.* 1992). These studies are consistent with acid-base and respiratory gas values in the fetal circulation and placental tissue during the first trimester (Jauniaux, Watson *et al.* 2001).

This low-oxygen environment in early pregnancy is essential for normal embryonic and placental development because the early conceptus has little protection against oxygen-generated free radicals (Poranen, Ekblad *et al.* 1996). Furthermore, studies have shown that early onset of maternal blood flow into the IVS is associated with early pregnancy loss (Hustin, Jauniaux *et al.* 1990; Jauniaux, Greenwold *et al.* 2003; Jauniaux, Zaidi *et al.* 1994), highlighting the importance of oxygen concentration on pregnancy success. In contrast, hypoxic exposure in late gestation due to insufficient invasion and remodelling of maternal vasculature can be detrimental and results in an increased risk of pregnancy complications, such as preeclampsia, intrauterine growth restriction and stillbirths (Kuzmina, Hubina-Vakulik *et al.* 2005).

The exact mechanism by which trophoblasts sense oxygen tension is currently unclear. However, several potential pathways have been identified. These pathways often utilize redox-sensitive transcription factors, of which the hypoxia inducible factor (HIF) family is the best characterized in trophoblasts.

#### 1.3.5.2.1 HIF-1

The adaptive response to low oxygen or hypoxia is regulated by a common oxygen-sensing pathway involving the formation of the hypoxia-inducible factor-1 (HIF-1) protein complex (Wang and Semenza 1993), although other transcription factors have also been reported to be involved, including NF- $\kappa$ B and AP-1 (Faller 1999). HIF-1 is a basic helix-loop-helix PAS transcription factor that binds to a short DNA consensus sequence (5' -RCGTG- 3') identified in the 5' flanking regions of hypoxia-induced genes called the hypoxia response element (HRE) (Jiang, Rue *et al.* 1996; Wang and Semenza 1995). HIF-1 binds to DNA as a heterodimeric complex composed of 2 subunits, HIF-1 $\beta$ , also known as ARNT (arylhydrocarbon receptor nuclear transferase, 91-94kDa) and HIF-1 $\alpha$  (120-130kDa) (Wang, Jiang *et al.*

1995a; Wang and Semenza 1995). Other isoforms of these subunits, ARNT2 (neuronal-specific) and ARNT3 (BMAL-1, circadian) and HIF-2 $\alpha$  and HIF-3 $\alpha$  have been described but their pattern of expression is more restricted.

Both HIF subunits are constitutively expressed, although under normoxic conditions it is thought that, HIF-1 $\alpha$  is rapidly degraded by increased ubiquitination and proteolysis through an interaction that appears to involve oxygen-dependent hydroxylation (Ivan, Kondo *et al.* 2001; Jaakkola, Mole *et al.* 2001; Masson, Willam *et al.* 2001; Yu, White *et al.* 2001) and interaction with the von Hippel-Lindau protein (Maxwell, Wiesener *et al.* 1999) (Figure 1.13). Under hypoxic conditions, the absence of oxygen prevents hydroxylation and therefore degradation of HIF-1 $\alpha$  (Huang and Bunn 2003), which then translocates from the cytoplasm to the nucleus, where it dimerises with HIF-1 $\beta$  to form the active HIF-1 complex (Wang, Jiang *et al.* 1995a; Wang, Jiang *et al.* 1995b) (Figure 1.13).

HIF-1 recruits transcriptional co-activators that induce expression of more than 20 target genes involved in energy metabolism including the glucose transporters *Glut-1* (Hayashi, Sakata *et al.* 2004) and *Glut-3*, glycolytic enzymes such as *lactate dehydrogenase*, *pyruvate kinase*, *tyrosine hydroxylase* and genes mediating angiogenesis, including *Vegf*, and apoptosis (Maxwell, Wiesener *et al.* 1999). Transcription of genes encoding IGFBP-1 (Tazuke, Mazure *et al.* 1998), IGFBP-2, IGFBP-3, IGF-II (Feldser, Agani *et al.* 1999; Kim, Bae *et al.* 1998; Steinbrech, Mehrara *et al.* 2000; Tucci, Nygard *et al.* 1998), IGF-I (Averbukh, Weiss *et al.* 1998), uPAR (Graham, Fitzpatrick *et al.* 1998) and TGF- $\beta$ 3 (Nishi, Nakada *et al.* 2004) are also induced by hypoxia in specific cell types. However, there is no data on the effect of oxygen concentration on IGF-II expression in the human placenta.

Other stimuli such as growth factors, cytokines and hormones may also be involved in regulating HIF-1 $\alpha$  expression. Indeed, investigations have shown that HIF-1 $\alpha$  protein stabilisation, HIF-1 DNA binding activity, and transactivation of target genes is induced under normoxic atmospheres by the treatment of human embryonic kidney cells or mouse embryonic fibroblasts with insulin, IGF-I and IGF-II (Feldser, Agani *et al.* 1999; Zelzer, Levy *et al.* 1998). More recently, IGF-I treatment of vascular tumor cells lead to the accumulation of HIF-1 $\alpha$  and HIF-2 $\alpha$ , with subsequent induction of the HIF target gene *Vegf*, by a process dependent on IGF1R (Catrina, Botusan *et al.* 2006). Further studies performed in human colon cancer cells have demonstrated that the effect of IGFs on HIF-1 $\alpha$  is a result of enhanced protein synthesis via effects on the translational machinery and not inhibition of von Hippel-Lindau-mediated ubiquitination (Fukuda, Hirota *et al.* 2002).



NOTE: This figure is included on page 46 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.13 Figure illustrating regulation of HIF-1 $\alpha$  protein stability.**

Under conditions of normal oxygen availability, HIF-1 $\alpha$  is hydroxylated and interacts with von Hippel-Lindau proteins (VHL), resulting in ubiquitination and subsequent proteolytic degradation in the cell cytoplasm. Under hypoxic conditions, HIF-1 $\alpha$  cannot be hydroxylated, and thus remains stable and can translocate to the nucleus where it dimerises with HIF-1 $\beta$  and then activates the transcription of genes containing hypoxia response elements (HRE).

Modified from Zagorska and Dulak, 2004.

As HIF-1 stimulates expression of the *Igf2* gene under hypoxia, HIF-1 $\alpha$  and IGF-II may be participating in an autocrine growth factor loop. Whether this growth factor cooperation exists during early pregnancy and orchestrates early placentation is currently unknown.

#### 1.3.5.2.2 Hypoxia and trophoblast

Oxygen concentration is a major influence on the balance between CTB proliferation and differentiation (invasion) during early placentation in humans. Culturing 5-8 week old placental villous explants in a hypoxic environment (2% O<sub>2</sub>; ~14 mmHg) stimulates trophoblast proliferation/outgrowth at tips of anchoring villi (Caniggia, Lye *et al.* 1997; Caniggia, Taylor *et al.* 1997; Genbacev, Zhou *et al.* 1997), fibronectin synthesis,  $\alpha$ 5 integrin expression and MMP-2 (Caniggia, Lye *et al.* 1997; Caniggia, Taylor *et al.* 1997) and FAK (MacPhee, Mostachfi *et al.* 2001; Seko, Takahashi *et al.* 1999) activity but inhibits upregulation of  $\alpha$ 1 $\beta$ 1 integrins typical of an invasive phenotype (Caniggia, Winter *et al.* 2000; Genbacev, Zhou *et al.* 1997). However, studies utilising immortalised trophoblast cells obtained from explant culture of human first trimester placenta (HRT-8/Svneo) have demonstrated that exposure to low oxygen (1% O<sub>2</sub>) stimulates trophoblast invasion by more than 40% due to reduced TIMP-1 and TIMP-2 secretion and increased uPAR expression (and therefore uPA cell surface-associated activity) compared with high (20%) oxygen (Canning, Postovit *et al.* 2001; Graham, Fitzpatrick *et al.* 1998).

Recent microarray and real time RT-PCR analyses of HTR-8/SVneo to either 20% or 1% oxygen have revealed that several genes that encode proteins involved in cell migration like cathepsin and protocadherin, angiogenesis, and apoptosis as well as anti-invasive factors including IGFBP-3, laminin, collagen, PAI-I, TIMP-3 are upregulated following hypoxia (Koklanaris, Nwachukwu *et al.* 2006), suggesting that a low oxygen environment may “fine-tune” trophoblast function.

Furthermore, hypoxia inhibits syncytial formation in human isolated mononuclear cytotrophoblasts (Alsat, Wyplosz *et al.* 1996), increases cell surface expression of  $\alpha$ v $\beta$ 3 integrin and migration of undifferentiated trophoblast cells and B16F0 melanoma cells (Cowden Dahl, Robertson *et al.* 2005). Additionally, hypoxia maintains the rat trophoblast cell line, Rcho-1, in a proliferative state and prevents their differentiation into giant cells (Gultice, Selesniemi *et al.* 2006). Interestingly, in a Rhesus monkey study where the abdominal aortic artery was constricted in early second trimester (day 116) to reduce uteroplacental blood flow, trophoblast invasion of the uterus and its vasculature by CTBs was increased (Zhou, Chiu *et al.* 1993).

Later studies using anti-sense oligonucleotides have demonstrated that the effect of hypoxia on human trophoblast function is mediated by HIF-1 $\alpha$  (Caniggia 2000). Indeed, both HIF-1 $\alpha$  and HIF-2 $\alpha$  protein are highly expressed by the STB, CTB and fetoplacental vasculature in humans (Nishi, Nakada *et al.* 2004; Rajakumar and Conrad 2000; Rajakumar, Doty *et al.* 2003; Rajakumar, Whitelock *et al.* 2001), consistent with the relative hypoxic environment of the placental intervillous space measured during the first trimester of pregnancy. Unexpectedly, hypoxic exposure upregulated the expression of the von Hippel-Lindau protein in CTB of first trimester human villous explants *in vitro* and it is expressed in proximal cell columns of human placenta *in vivo* (Genbacev, Krtolica *et al.* 2001). This suggests that other factors may be involved in the regulation of HIF-1 $\alpha$  abundance at the feto-maternal interface.

Later in gestation, studies performed on 10 to 12 weeks old villous explants revealed that relatively high oxygen atmospheres (8% and 20%) inhibit CTB proliferation but promote CTB invasion by the increased expression of  $\alpha$ 1 integrin as compared with low oxygen (2%) (Genbacev, Joslin *et al.* 1996).

From these studies, it appears that there are conflicting data on the role of oxygen in the regulation of trophoblast function. One train of thought is that early in the first trimester (prior to 8 weeks) a low oxygen environment causes CTBs to proliferate but prevents their differentiation along the invasive pathway (Caniggia, Winter *et al.* 2000; Caniggia and Winter 2002; Genbacev, Zhou *et al.* 1997). This is on the contrary to other researchers, which have demonstrated that low oxygen atmospheres promotes trophoblast invasion (Graham, Postovit *et al.* 2000). This may reflect a difference in the composition of culture media used, as the latter investigation supplemented the media with plasminogen, a critical mediator of EVT invasion *in vivo* (Graham, Postovit *et al.* 2000).

### 1.3.5.3 Transforming growth factor- $\beta$

In humans, TGF $\beta$  is a 25kDa dimeric protein that exists in several isoforms, with TGF $\beta$ 1 to  $\beta$ 3 expressed in mammals. The TGF $\beta$  isoforms share 70-80% structural homology with each other and are highly conserved between mammalian species. All three TGF $\beta$  isoforms are synthesised at the feto-maternal interface during the first trimester (Ando, Hirahara *et al.* 1998; Caniggia, Grisaru-Gravnosky *et al.* 1999; Dungy, Siddiqi *et al.* 1991) and are believed to be major factors regulating CTB behaviour. Although members of the TGF $\beta$  gene family are known to have both stimulatory and inhibitory effects on cell growth, TGF $\beta$  itself is commonly thought to inhibit cell proliferation and invasion (Bischof 2001). More research is warranted to determine the specific mechanisms by which TGF $\beta$  influences placentation and how it is regulated during early pregnancy.

#### 1.3.5.3.1 *Activation of TGF $\beta$*

TGF $\beta$  is secreted in a latent pro-form (LTGF $\beta$ ) that is complexed with a 110kDa latency-associated peptide (LAP) from which biologically active TGF $\beta$  is released that can subsequently interact with specific cell surface receptors. Studies in human monocytes have revealed that activation of TGF- $\beta$ 1 involves the binding of LTGF- $\beta$ 1 to the IGF2R (due to M6P residues in LAP) (Dennis and Rifkin 1991; Kovacina, Steele-Perkins *et al.* 1989) (Figure 1.14) which then complexes with the uPAR on the cell surface (Godar, Horejsi *et al.* 1999). The binding of uPA to uPAR allows uPA to cleave plasminogen into plasmin. The latter then cleaves the LAP and allows release of TGF- $\beta$ 1. As TGF- $\beta$  has been shown to stimulate the production of the plasminogen activator inhibitor-1 (Fitzpatrick and Graham 1998), it has been proposed to regulate its own activity.

#### 1.3.5.3.2 *TGF $\beta$ receptors*

Active TGF $\beta$  binds to the specific cell surface receptors, TGF $\beta$  type 1 receptor (TGF $\beta$ IR), TGF $\beta$  type 2 receptor (TGF $\beta$ IIR), betaglycan and endoglin (Wrana, Attisano *et al.* 1994). These are expressed by the placenta during pregnancy (Ciarmela, Florio *et al.* 2003; Mitchell, Fitz-Gibbon *et al.* 1992; St-Jacques, Forte *et al.* 1994) and the latter two can inhibit trophoblast proliferation and invasion, independent of TGF- $\beta$  (Caniggia, Taylor *et al.* 1997; Xu, Guimond *et al.* 2002). TGF $\beta$ IR and TGF $\beta$ IIR are serine kinases and upon ligand binding, form a heterodimeric receptor complex, which then regulates gene expression through the Smad family of proteins.

#### 1.3.5.3.3 *TGF $\beta$ and trophoblast function*

TGF $\beta$ 1 and TGF $\beta$ 2 are synthesised primarily by the decidua and to a lesser extent, the STB and extravillous CTBs throughout gestation (Graham, Lysiak *et al.* 1992; Lysiak, Han *et al.* 1993; Lysiak, Hunt *et al.* 1995). TGF $\beta$ 1 is a negative regulator of first trimester CTB cell proliferation (Graham and Lala 1992) migration (Irving and Lala 1995) and invasion (Graham and Lala 1991; Graham and Lala 1992). TGF- $\beta$ 1 is also known to inhibit steroidogenesis in human trophoblasts (Luo, Yu *et al.* 2002) and promotes syncytium formation in isolated first trimester trophoblast primary cultures by increasing surface expression of cadherin-11 (Getsios, Chen *et al.* 1998). Later in pregnancy, TGF $\beta$  may play a role in promoting placental differentiation, as TGF $\beta$  exposure stimulates murine trophoblast stem cell line, SM10, differentiation into labyrinthine trophoblast, *in vitro* (Selesniemi, Reedy *et al.* 2005).

NOTE: This figure is included on page 50 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.14 Figure demonstrating activation of transforming growth factor (TGF)- $\beta$ 1 at the type 2 IGF receptor (IGF2R).**

Latent TGF- $\beta$ 1 bound to IGF2R, can be activated through a simultaneous interaction of IGF2R with the urokinase plasminogen activator receptor (uPAR) on the cell surface. Urokinase plasminogen activator (uPA) bound to the uPAR, cleaves plasminogen into plasmin, which then cleaves the latency associated peptide and allows release of active TGF- $\beta$ 1.

Diagram from Godar *et al.*, 1999, modified by Roberts, CT.

TGF $\beta$ -1 inhibits trophoblast CTB invasion by inducing the expression of TIMPs and reducing uPA activity (Graham and Lala 1991; Khoo, Bechberger *et al.* 1998). TGF $\beta$ -1 also increases the production of oncofetal fibronectin, laminin and collagen (Chen, Yang *et al.* 2005; Feinberg, Kliman *et al.* 1994) and PAI-1 (Graham 1997), up-regulates E-cadherin expression (Karmakar and Das 2004), increases surface expression of  $\alpha$ 1,  $\alpha$ 5 and  $\alpha$ v integrins by first trimester trophoblast and inhibits MMP-9 activation and synthesis by CTBs (Meisser, Chardonens *et al.* 1999). Interestingly, *in vitro* studies of murine blastocysts demonstrated that TGF- $\beta$ 1 facilitated trophoblast outgrowth through autocrine release of parathyroid hormone-related protein (Nowak, Haimovici *et al.* 1999), indicating that there may be differentiation stage and/or species specificity for TGF- $\beta$  actions.

Like TGF- $\beta$ 1 and TGF- $\beta$ 2, TGF- $\beta$ 3 is known to inhibit the early events of trophoblast differentiation along the invasive pathway, however, unlike the other TGF- $\beta$  isoforms, TGF- $\beta$ 3 is expressed by CTB, STB and villous mesenchyme during early placentation (6-8 weeks) and rapidly declines at the end of the first trimester (by 9-10 weeks) (Caniggia 2000; Caniggia, Grisaru-Gravnosky *et al.* 1999). Using electrophoretic mobility shift assays, it has been shown that this temporal regulation is mediated by HIF-1 interacting with a HRE in the *Tgf $\beta$ 3* gene core promoter (Nishi, Nakada *et al.* 2004). Caniggia and associates, have speculated that TGF $\beta$ 3 mediates the inhibitory effects on CTB differentiation at 5-8 weeks of gestation (Caniggia 2000).

Recently, the serine proteases, HtrA1 and HtrA3 were demonstrated to inhibit TGF- $\beta$  signaling by an unknown mechanism that required the proteolytic activity of HtrA (Oka, Tsujimoto *et al.* 2004; Tocharus, Tsuchiya *et al.* 2004). Both HtrA1 and HtrA3 are highly expressed in the human placenta (Nie, Hampton *et al.* 2003), with HtrA3 localized exclusively to the decidua of mice (Nie, Li *et al.* 2006). Both HtrA proteins possess an IGF-binding site and it has been speculated that the IGFs themselves may activate this protease family (Zumbrunn and Trueb 1996) as recent reports indicate that HtrA1 binds to, but does not cleave, IGF-I *in vitro* (Tocharus, Tsuchiya *et al.* 2004). Conversely, TGF $\beta$  may in turn regulate IGF-II through its regulatory impacts on the abundance of IGFBP-4 protease, PAPP-A (Durham, Riggs *et al.* 1994; Ortiz, Chen *et al.* 2003). Interestingly, TGF $\beta$ -1 inhibits IGF-II expression in cultured osteoclasts (Gabbitas, Pash *et al.* 1994).

## 1.4 Conclusion, hypotheses and aims

Given the pro- and anti-invasive effects of the IGFs and TGF- $\beta$  on trophoblast function, respectively, as well as the abundance of these proteins at the feto-maternal interface, further studies are required to determine their interactions as it is likely that together they orchestrate placentation. Indeed, there is considerable overlap between the molecules that both the IGFs and TGF- $\beta$  interact with, including the IGF2R. Furthermore, studies suggest that a low oxygen environment is important for early cytotrophoblast proliferation and invasion of the maternal decidua. However, later in the first trimester, when maternal blood perfuses the placental IVS, an increase in oxygen concentration causes CTBs to become less proliferative and more invasive. As *Igf2* gene expression is induced by HIF-1 under a hypoxic environment in some cells, and IGF-II can stabilise HIF-1 $\alpha$  under normoxic conditions, oxygen concentration at the feto-maternal interface may also influence the putative IGF and TGF- $\beta$  interactions.

Further elucidation of the effects and mechanisms of action of maternal IGF-I and IGF-II regulation of fetal growth are required. IGFs may affect placental functional development, which can have direct impacts on fetal growth by influencing substrate delivery to the fetus. IGFs, however, may also indirectly influence fetal growth through modulating maternal adaptation to pregnancy, via synthesis of modulatory hormones, which influence nutrient utilization and partitioning between the mother and fetus.

**Therefore it is hypothesised that:**

- 1) CTB expression of IGF-II is induced by a low oxygen environment, which promotes CTB proliferation, migration and subsequent invasion by altering CTB gene expression and inhibiting TGF- $\beta$ 1 activation.
- 2) Maternal administration of IGF-I or IGF-II in early pregnancy promotes growth of the placenta with increased functional capacity to transfer nutrients to the fetus and therefore enhances fetal development and increases pregnancy success.

**The general aims of this project are:**

- 1) To determine the effect and interaction of IGF-II with oxygen and TGF- $\beta$ 1 in controlling the balance between CTB proliferation and differentiation/invasion during early pregnancy, *in vitro*.
  
- 2i) To investigate the acute effects of maternal IGF-I or IGF-II treatment during early to mid pregnancy on placental transfer capacity, maternal nutrient utilization and fetal growth in mid gestation.
  
- 2ii) To investigate the sustained effects of maternal IGF-I or IGF-II treatment during early to mid pregnancy and the contribution of the IGF2R on placental development, maternal body composition and fetal growth near term.
  
- 2iii) To investigate the sustained effects of maternal IGF-I or IGF-II treatment during early to mid pregnancy and the contribution of the IGF2R on placental transport to the fetus, fetal substrate utilization and nutrient partitioning near term.



## *Chapter 2 : Materials and Methods*

## 2.1 First trimester human chorionic villous explant culture

This study was approved by the Human Ethics Committee of the Women's and Children's Hospital, Adelaide, South Australia and all women gave informed consent. First trimester (7-12 weeks gestation) human placenta was obtained immediately following elective terminations of pregnancy by dilatation and curettage performed in the Women's and Children's Hospital. Gestational age was determined by last menstrual period.

### 2.1.1 Solutions

#### 2.1.1.1 Villous collection media

Collection media was prepared the day prior to placental sample collection and stored at 4°C. This media consisted of HEPES buffered DMEM/F12 (GIBCO BRL, Grand Island NY, USA) supplemented with 10% heat inactivated fetal calf serum (CSL, Australia) and 40 ng/ml gentamycin (GIBCO BRL, Grand Island NY, USA).

#### 2.1.1.2 Villous serum-free culture media

Serum-free culture media was prepared at least 18h prior to sample collection and divided into three flasks and equilibrated in either 20% (5% CO<sub>2</sub> in 95% air), 1% or 5% oxygen (both in modular chambers, 94% or 90% N<sub>2</sub> and 5% CO<sub>2</sub>, respectively) at 37°C. The media consisted of DMEM/F12 (GIBCO BRL, Grand Island NY, USA), supplemented with 2% Nutridoma NS (filter sterilised, Boehringer Mannheim Pty, Australia), 0.04 mM L-glutamine, 20 ng/ml plasminogen and 20 µl/ml antibiotic mix (GIBCO BRL, Grand Island NY, USA), comprised of 200µg/ml streptomycin, 0.5 µg/ml amphotericin and 200 units/ml penicillin.

### 2.1.2 Preparation and culture of explants

Placental villous tissue was washed using sterile cold phosphate buffered saline (PBS, Dulbeccos Mg<sup>2+</sup> Ca<sup>2+</sup> free) to remove blood and decidua and transported on ice to the laboratory in villous collection media (refer to 2.1.1.1).

Placental tissue was rinsed and aseptically dissected to remove fetal membranes and any residual decidual tissue in serum-free culture media (refer to 2.1.1.2). Placental tissue was then teased and dissected into small fragments of placental villi (10-20 mg wet weight) that were comprised of a stem

villus with about 4-6 floating branched villi. From each placenta, 48 villous explants were established by plating a single villous fragment per culture well of 4 well plates (Becton Dickinson, USA) that had been pre-coated for a minimum of 30 minutes at 37°C in a humid incubator with 20 µl of growth factor reduced Matrigel (GIBCO BRL, Grand Island NY, USA) diluted 1:1 with serum-free culture media. Explants were then assigned to six treatment groups, 20%, 5% or 1% oxygen with or without exogenous IGF-II, achieving 8 explants per treatment, per placenta. Plated villous pieces were then covered with 15 µl of Matrigel, diluted 1:1 with culture media and incubated for at least 25 minutes at 37°C in 5% CO<sub>2</sub> in air to allow attachment.

200 µl of serum-free culture media that had been equilibrated to 20% (5% CO<sub>2</sub> in 95% air), 5% or 1% (both in modular chambers, 94% or 90% N<sub>2</sub> and 5% CO<sub>2</sub>, respectively) oxygen for at least 18 hours was then added to 16 explants per oxygen concentration and incubated overnight at 37°C in the appropriate oxygen environment.

The next morning (~15h), by which time the villous tips had adhered to the bottom of the well, 800 µl of the corresponding 20%, 5% or 1% oxygenated culture media was added per well. IGF-II at a final concentration of 125 ng/ml (~17 nmol) (recombinant human IGF-II, GroPep, Australia) or equivalent vehicle PBS volume was then added to the appropriate wells. Explants were returned to the same oxygen atmospheres and were maintained for 5 days, without changing the media.

On day 6 of culture, villous explants were photographed on a NIKON inverted microscope using an Olympus DP12 digital camera, immediately before the culture media was removed and frozen for analysis of IGF-II and TGFβ1 protein. Total RNA extraction was performed on the explanted villous tissue (see below).

To observe the morphological integrity and growth of the villous explants over the 6 days in culture, some explants were photographed daily and were not included in further analyses. Additional villous explants were established in wells containing thermanox coverslips beneath the Matrigel (Nunc Brand Products, USA) and fresh frozen in OCT following removal of the conditioned media for immunohistochemical localisation of proteins.

### ***2.1.3 Analysis of villous explant outgrowth morphology***

Digital photos of explants were assessed for villous tip outgrowth by the researcher blinded to treatment. EVT cell outgrowth and migration were quantitated using the ratio of extravillous cytotrophoblast outgrowths/villous tip, where the numerator, EVT outgrowths, represents the number of EVT columns sprouting from the villous tips and the denominator represents the total number of villous tips in a single explant culture. An arbitrary scale of 0-3 was used to assess both the extent of outgrowth/proliferation at the distal end of villous tips and the distance to which cytotrophoblast had detached and migrated away from the villous tip and into the surrounding matrix. Additionally, the phenotype of extravillous cytotrophoblasts was classified using arbitrary scores of 1 or 3, as being predominantly round or dendriform, respectively, with a dendriform score attributed to cells of a migratory phenotype. A score of 2 was assigned to explants displaying a 50:50 mixture of round and dendriform extravillous cytotrophoblasts.

### ***2.1.4 Analysis of villous explant gene expression***

#### ***2.1.4.1 Villous explant RNA extraction***

Immediately following the removal of the villous conditioned media, total RNA was isolated from villous explants using a Trizol-based approach according to the manufacturer's protocol for cells grown in a monolayer (GIBCO BRL, Grand Island NY, USA).

Briefly, to each villous explant, 300  $\mu$ l TriZol (GIBCO BRL, Grand Island NY, USA) was added and incubated for 5 minutes at room temperature. The undigested villi were removed with forceps, the TriZol containing lysed EVT's from 4 wells for each treatment group, were pooled to achieve two tubes containing extracted EVT outgrowths. 240  $\mu$ l of chloroform was added to each pool, shaken for 15 seconds and incubated at room temperature for 3 minutes. Samples were then centrifuged for 15 minutes at 12,000 g at 0-4°C to separate the RNA from other cellular components. The top, clear and aqueous layer was then discarded. 600  $\mu$ l of isopropyl alcohol was then added to each tube to precipitate the RNA and then incubated for 10 minutes at room temperature. To pellet the RNA, the tubes were then centrifuged for 15 minutes at 12,000 g at 0-4°C. The supernatant was removed and the pellet was allowed to air dry for 25 minutes before 25  $\mu$ l of molecular grade water was added to each tube and vortexed for 1 minute. RNA was then pooled for each treatment group, allowing one tube of RNA in 50  $\mu$ l to be achieved per treatment, per placenta.

#### 2.1.4.2 Determination of RNA quantity and purity

The quantity and purity of RNA in the sample was determined using a Spectrophotometer (DU-50, Beckman Instruments, CA). Samples were considered to be sufficiently pure if the ratio of OD<sub>260</sub>:OD<sub>280</sub> was > 1.7. The quantity of RNA was then calculated using the following equation: OD<sub>260</sub> x 40 x dilution factor = RNA µg/ml. Each tube was then frozen at -80°C for subsequent reverse transcription and Real Time polymerase chain reaction (PCR).

#### 2.1.4.3 Reverse Transcription

Reverse transcription was performed in an Applied Biosystems Conventional Thermocycler (Applied Biosystems, Warrington, UK). Aliquots of 20 µl containing 2 µg of RNA from each sample were reverse transcribed. RNA was incubated with 2 µl of random hexamer primers (100 µg/ml; GeneWorks, SA, AUS) at 65°C for 10 minutes. Samples were cooled on ice for at least 3 minutes before 18 µl of a master mix containing 5x buffer, 100 mM DTTs, 5000 units/µl Expand RTase (all three from Boehringer Mannheim Pty, Australia) 10 mM Ultrapure dNTPs (Amersham Pharmacia Biotech, New Jersey, USA) was added per sample. Samples were allowed to stand on ice for 3 minutes before they were incubated for 10 minutes at 30°C, followed by 45 minutes at 42°C and then for 2 minutes at 95°C. The samples were then cooled on ice and the cDNA was stored at -20°C.

#### 2.1.4.4 Primer Design

Using Primer Express 2.0 software (Applied Biosystems, CA) and Genbank DNA and mRNA sequences, oligonucleotide primers for human *Igf2*, *Igf1r*, *Igf2r*, *Tgfβ1*, *Upa*r were designed within the mature peptide region (Table 2.1). Where possible, primers were designed such that the generated amplicon overlapped an exon-intron boundary. Standard desalted purity primers were constructed by Sigma Genosys (Sigma Genosys, AUS).

#### 2.1.4.5 Real Time Polymerase Chain Reaction (PCR)

Quantitative PCR was performed in real-time, on an Applied Biosystems 5700 Real Time Thermocycler (GeneAmp 5700, Applied Biosystems, Warrington, UK). The PCR reaction mix consisted of 10 µl 1x SYBR gene PCR master mix (Applied Biosystems, Warrington, UK), 2 µl cDNA template, 1 µl of each forward and reverse primer and 6 µl molecular grade water. The PCR cycle conditions were one cycle of 50°C for 2 minutes and 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was analysed in duplicate and plates were set up such that representatives for

Table 2.1 Primer sequences designed for Real Time PCR

Gene	Nucleotide position	Primer sequence (5'-3')	Amplicon size (bp)	Genbank Accession #
<i>Igf2</i>	7644 (fwd)	CCCCTCCGACCGTGCT	90-100	X03562
	7987 (rev)	TGGACTGCTTCCAGGTGTCAT		
<i>Igf2r</i>	6333 (fwd)	GCAGAAGCTGGGTGTCATAGG	88	AF348209
	6420 (rev)	CACGGAGGATGCCGGTCTTAT		
<i>Igf1r</i>	1245 (fwd)	CTTCCTAAAAACCTTCGCCTCAT	142	NM00875.2
	1386 (rev)	GTACATTTCCCTGCTTTGATGGT		
<i>Upar</i>	693 (fwd)	CGAGGTTGTGTGTGGGTTAGAC	71	NM002659
	763 (rev)	GGCTTCGGGAATAGGTGACA		
<i>Tgfβ1</i>	1786 (fwd)	CAAGGGCTACCATGCCAACTT	104	X02812.1
	1889 (rev)	CCGGGTTATGCTGGTTGTACA		
<i>18s</i>	56 (fwd)	AGAAACGGCTACCACATCCAA	91	AF176811
	199 (rev)	CCTGTATTGTTATTTTCGTCACCT		

Both forward (fwd) and reverse (rev) primers are shown, with amplicon size denoted in base pairs (bp).

each treatment per placenta were run on each plate. To reduce inter-assay variation, the house keeping gene, 18s was run for each sample on the same plate as the gene of interest.

#### *2.1.4.6 Detection of PCR products*

The PCR products were separated by size using gel electrophoresis. PCR products (20  $\mu$ l) containing 4  $\mu$ l of 6x loading buffer were run on a 2% agarose gel (Promega, WI, USA) containing ethidium bromide (final concentration of 0.5 mg/ml, Sigma, MO, USA) for 45 minutes at 85 V. pUC19 (GeneWorks, SA, AUS) was used as a size marker. The gel was visualised over a UV light box (UVP, San Gabriel, CA, USA) to detect bands of PCR products and photographed using a digital camera (DC120, Kodak, Eastman, Rochester, USA).

#### *2.1.4.7 Quantification of mRNA levels*

Fluorescence was measured at the end of each cycle, and after 40 cycles, a profile of fluorescence versus cycle number was obtained. A fluorescence threshold of 0.14 was set, which lay in the exponential phase of amplification. The cycle number at which the amplification of the product crossed this threshold was determined and designated as the cycle threshold (C<sub>T</sub>). The quantity of amplified PCR product was determined by normalising the expression of the gene of interest to the house keeping gene, 18s, and then expressing it relative to a calibrator using the  $2^{-\Delta\Delta C_T}$  formula as described in the ABI Prism 5700 User Bulletin (Liu and Saint 2002a; Liu and Saint 2002b). The mean of the 20% oxygen without exogenous IGF-II treatment group was designated as the calibrator and was assigned an expression level of 100%. 18s did not significantly vary across the six treatments studied. The efficiency of each PCR reaction was determined by regression analysis of the sample's fluorescence at each cycle and all reactions occurred at a minimum of 73% efficiency.

#### *2.1.4.8 Optimisation of Real Time PCR reactions*

The optimum concentration of primers were initially determined in preliminary experiments with serial dilutions of primer pairs and a quality control uncultured first trimester human villous cDNA sample. The lowest primer concentration at which the threshold cycle (C<sub>T</sub>) of the sample was most different to the C<sub>T</sub> of the non-template control (noise) was selected.

The optimum cDNA dilution for each primer pair was initially determined in preliminary experiments with a range of cDNA concentrations. The cDNA concentration at which the C<sub>T</sub> increase was linear and most

different to the  $C_T$  of the non-template control was selected. It was determined that a 1 in 5 dilution was required for quantitative analysis of 18s, whereas neat cDNA was used for analysis of all other genes.

The absence of primer dimers and alternative PCR products in the PCR product was revealed by a single dissociation peak and confirmed by PCR product visualisation on 2% agarose gels (Promega, WI, USA) containing ethidium bromide (final concentration of 0.5 mg/ml, Sigma, MO, USA) for 45 minutes at 85 V.

### *2.1.5 Localization of protein in villous explants*

To localise cytokeratin-7 and IGF-II proteins, villous explants grown for 6 days on Matrigel-coated thermanox coverslips were fresh frozen in OCT (Tissue-Tek; Sakura, USA) and snap frozen in isopentane cooled by liquid nitrogen. Serial 5  $\mu$ m sections were cut and mounted on silane-coated slides (3-aminopropyltriethylsilane, Sigma, Australia) and a three-step immunohistochemistry technique was employed.

Briefly, sections were fixed in 96% ethanol and washed three times in PBS (Dulbeccos  $Mg^{2+}$   $Ca^{2+}$  free) for 5 minutes. The sections were then incubated for 10 minutes at room temperature with serum-free protein block (Dako, Australia) before overnight incubation at 4°C with primary antibody, mouse anti-human cytokeratin-7 (1:500 dilution in PBS containing 1% BSA and 10% normal human serum, Clone OV-TL 12/30, Dako, Australia) or goat anti-human IGF-II (1:50 dilution in PBS containing 10% serum-free Dako protein block R and D Systems, MN, USA).

The following day, the sections were rinsed in Milli-Q water and washed three times in PBS. Bound primary antibody was localised using a biotinylated rabbit anti mouse IgG F(ab')<sub>2</sub> fragment (Dako, Australia) or rabbit anti-goat IgG F(ab')<sub>2</sub> fragment (Dako, Australia), for immuno-localisation of cytokeratin-7 or IGF-II, respectively. Sections were incubated for 45 minutes at 4°C. The sections were then rinsed in milli-Q water and washed three times in PBS and a streptavidin-horseradish peroxidase (Rockland, USA) was applied for 30 minutes at 4°C. After the sections were washed as described above, the site where the antibodies bound was visualised using diaminobenzidine (Sigma, Australia; 5 mg/ml in 0.05 M Tris-HCl, pH 7.2) plus 0.02% hydrogen peroxide for 10 minutes at room temperature and before light counterstaining in haematoxylin. Negative controls were incubated with the primary antibody diluent on its own. Immuno-histochemical staining was captured using a digital camera under an Olympus DP10 camera with an Olympus BX-2 microscope.



### ***2.1.6 Analysis of villous explant protein secretion by Enzyme-linked Immunosorbant Assay***

#### ***2.1.6.1 TGF- $\beta$ 1 protein***

Villous-conditioned media were assayed for active and total TGF- $\beta$ 1 protein using a Human Quantikine TGF- $\beta$ 1 enzyme-linked immunosorbant assay (ELISA) (R and D Systems, MN, USA), following the manufacturer's instructions. Samples were run in duplicate and media blanks were run on each plate. The concentration of total and active TGF- $\beta$ 1 protein present in the media used to culture the villous explants was ~16.5 pg/ml and undetectable, respectively, with the minimum detectable concentration of TGF- $\beta$ 1 is < 7 pg/ml. Media blanks were subtracted from the unknown samples and TGF- $\beta$ 1 active and total protein concentrations were determined using a four-parameter logistic regression curve. The mean coefficient of variation for each assay was 2.66%.

#### ***2.1.6.2 IGF-II protein***

Villous-conditioned media were assayed for IGF-II protein using the Active IGF-II ELISA (Diagnostic Systems Laboratories Inc, Texas, USA) following the manufacturer's instructions. This ELISA kit incorporated an acid-ethanol extraction step to dissociate the IGF-II protein from any binding proteins. Samples were run in duplicate and media blanks were run on each plate. The concentration of IGF-II protein present in the media without exogenous IGF-II was less than the minimum detectable concentration of IGF-II for the assay, which was 24 ng/ml. IGF-II protein concentrations were determined from a standard curve. The mean coefficient of variation for each ELISA was 6.1%.

### ***2.1.7 Statistics***

Data generated from morphological studies were assessed using a linear mixed model repeated measures ANOVA with Sidak Post Hoc Tests with the placenta as a subject and each well of a particular treatment, as the repeated measure. ELISA data were analysed by a multi-variate ANOVA with Bonferoni Post Hoc Test (SPSS Software, Chicago). Data generated from Real Time PCR analyses were normalised by ranking  $\Delta$ Cts and compared for each gene using Kruskal Wallis followed by Mann-Whitney U Tests. Pearson's bi-variate correlation analysis was used for associating gene and protein expression with villous explant morphology. Differences between treatments or correlations considered to be significant were when  $P < 0.05$ .

## 2.2 Animals and treatments

This study was approved by the University of Adelaide, Animal Ethics Committee. Virgin guinea pigs (IMVS coloured strain, ~500g, 3-4 months old) were purchased from the Gilles Plains breeding facility and housed individually in the University of Adelaide Medical School Animal House in a room with 12 : 12 h light : dark cycle. Guinea pigs were provided with food (Milling Industries Stockfeeds, Murray Bridge, SA, Australia) and water supplemented with vitamin C (400mg/L) ad libitum.

### 2.2.1 Matings

Females were examined daily for oestrus, indicated by a ruptured vaginal membrane (complete oestrous cycle lasts ~15 days), and mated naturally with a male. The day a copulatory plug was observed or in the absence of a plug, the day after females were housed with males, was designated as day 1 of pregnancy. From 2 weeks prior to mating, body weight was monitored three times weekly. At mating, females were assigned to one of three treatment groups of similar mean weight (~650 g). Animals were handled in accordance with University of Adelaide Animal Ethics Committee.

### 2.2.2 Surgery

Surgery was performed on female guinea pigs twice, first to insert mini osmotic pumps and secondly to catheterise the carotid artery and jugular vein. All instruments utilised in surgical procedures were sterilised prior to use by washing in water treated with pyroneg detergent, sonication and then autoclaving or soaking them in 70% ethanol (v/v). All instruments were immersed in chlorhexidine (75% methanol, 10% chlorhexidine (Chlorhex C, Jurox, Australia) and 15% Milli-Q) during the surgical procedure to maintain sterility. All drapes, cotton gauze, sutures and cotton tips were packaged and autoclaved for use on the day. Animals were anaesthetised with atropine sulphate (0.05 mg/kg, sc; Apex Laboratories, Australia), xylazine hydrochloride (4 mg/kg, im, Troy Laboratories, Australia), ketamine hydrochloride (25 mg/kg, ip; Troy Laboratories, Australia) and locally with lignocaine hydrochloride (Troy Laboratories, Australia), unless otherwise stated. The area where the incision was to be made (see below) was shaved free of hair and then wiped clean with swabs soaked in Betadine (AnalaR, Australia), 70% ethanol and Betadine, again. After a surgical procedure, guinea pigs were kept warm at 37°C during recovery. Mothers were killed, following an over-night fast, by overdose of sodium pentobarbitone (Lethobarb; Virbac, Australia) on day 35 or day 62 of pregnancy (term ~70 days).

### *2.2.2.1 Minipump insertion*

On day 20 of pregnancy, females were anaesthetised and a small horizontal incision was made in the skin, mid-way down the animal's back and slightly off-centre to expose the muscle layer. With a small artery clamp inserted into the incision with the curve faced down, a vertical pocket that reached up between the shoulder blades was made by gently opening and closing the clamp. An osmotic minipump (Alzet 2002, Alzet, California) was then inserted into the pocket and positioned away from the incision. Osmotic minipumps had previously been prepared to deliver vehicle (0.1M acetic acid) or 1mg/kg/day IGF-II, Leu<sup>27</sup>-IGF-II or IGF-I (human recombinant protein, GroPep Pty Ltd., Australia) for 18 days at a flow rate of 0.51  $\mu\text{l/h}$ . The mean fill volume of the osmotic minipumps in this experiment was 233  $\mu\text{l}$  (221  $\mu\text{l}$  when accounting for 5% residual volume), which means that 632  $\mu\text{g}$  of IGF-I or IGF-II per day was delivered based on weight at mating. The surgical incision was then closed using 9 mm wound clips (Becton Dickinson, USA).

### *2.2.2.2 Catheterization*

Mothers that were scheduled to be killed on day 62 of pregnancy were catheterised two days prior to post-mortem. Animals were anaesthetised according to weight on day 20 of pregnancy, as described above (refer to 2.2.2), however with larger doses of xylazine hydrochloride (6 mg/kg, im, Troy Laboratories, Australia) and ketamine hydrochloride (75 mg/kg, ip; Troy Laboratories, Australia). An incision of 1-2 cm was made mid way between the head and sternum, to the left of the trachea on the ventral side of the neck. The external jugular vein and common carotid artery were located and vascular catheters were inserted. Catheters were fastened to the vessels and then to the inside of the neck using silk suture (Dysilk black braid 3/0, Dynek, Australia). Catheters were then passed through to the back of the neck and exteriorised through the skin and animals allowed to recover. Catheters were flushed twice daily with 250 U/ml sterile heparinised physiological saline to maintain patency.

### *2.2.3 Radioactive analogue administration and post-mortem*

On day 35 or 62 of pregnancy (term ~70 days) following an overnight fast, guinea pigs were administered a mixture of [<sup>3</sup>H]-methyl-D-glucose (MG) and methyl [<sup>14</sup>C]-amino-isobutyric acid (AIB) (both from Amersham, UK) in physiological saline in a single bolus via catheter. To each dam, we aimed to treat with 100  $\mu\text{Ci}$  / kg of MG and 10  $\mu\text{Ci}$  / kg of AIB, but determined the precise dose actually administered by weighing syringes containing each isotope before and after the preparation of the isotopic mixture. The total administered volume of the isotope mixture was 500  $\mu\text{l}$  for each animal.

Maternal blood was collected in heparinized tubes 20 minutes following radioactive analogue administration and a final 10-20 ml blood sample was taken before animals were then killed by overdose of sodium pentobarbitone (Lethobarb; Virbac, Australia). This chosen 20 minute time-point was based on a previous, similar study assessing placental AIB and MG transport and blood flow, in the unstressed, awake pregnant guinea pig (Jansson and Persson 1990), where substrate analogues were detected in the fetus and placenta 25 minutes following administration to the mother. In animals killed on day 62 with patent catheters, blood was sampled (1 ml) every 2 minutes following radioactive analogue administration, with the catheter flushed with 1ml of heparinised saline, at each time point.

Viable and resorbing implantation sites were enumerated and the uterus and its contents, viable fetuses and placentae were weighed. A 3 mm mid-sagittal placental slice was fixed in paraformaldehyde (PFA, 4% paraformaldehyde / 2.5% polyvinylpyrrolidone-40 in 70 mM PBS (Dulbeccos Mg<sup>2+</sup> Ca<sup>2+</sup> free)) for histology and morphology to estimate structural correlates of placental function. Remaining halves of placental tissues were snap frozen for measurement of AIB and MG content (see below) and RNA or protein analyses (not performed for the present thesis). In fetuses of mothers killed on day 62, fetal biparietal diameter, abdominal circumference and crown to rump length were measured and whole blood samples were collected by cardiac puncture in heparinized tubes.

The following tissues of the mother and all fetuses from day 62 of pregnancy were then excised and weighed to determine the absolute and relative weights of adrenals, *kidneys*, pancreas, *liver*, *spleen*, *heart*, *brain*, *lungs*, gastrointestinal tract, reproductive tract, biceps, *triceps*, *gastrocnemius* and soleus muscles and *retroperitoneal*, *perirenal* and *interscapular* adipose tissues. Skin and carcass weights of the dams and carcass weights of the fetuses were also recorded. Those tissues printed in italics were snap frozen in liquid nitrogen and stored at -80°C for determination of MG and AIB content. Fetal and maternal whole blood samples were centrifuged at 2,500 rpm for 15 min at 4°C to recover plasma, which was stored at -20°C.

## ***2.2.4 Analysis of circulating metabolites and hormones***

### ***2.2.4.1 Assay of plasma glucose***

The quantitative determination of plasma glucose was performed with a COBAS MIRA automated sample system using the Glucose HK assay kit, with the C.f.a.s Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, Australia). This was performed in a single assay with the intra-assay coefficient of variation < 1%.

#### 2.2.4.2 Assay of plasma free fatty acids

The quantitative determination of plasma free fatty acids was performed with a COBAS MIRA automated sample system using the WAKO NEFA-C Free Fatty Acid Kit assay kit (NovoChem, Australia), with the Quality controls QCS-1 and 2 (Biorad, Australia). This was performed in a single assay with the intra-assay coefficient of variation < 1%.

#### 2.2.4.3 Assay of plasma cholesterol

The quantitative determination of plasma cholesterol was performed with a COBAS MIRA automated sample system using the Cholesterol CHOD-PAP assay kit, with the C.f.a.s Calibrator and quality controls: Precinorm U and Precipath U (Roche Diagnostics, Australia). This was performed in a single assay with the intra-assay coefficient of variation < 1%.

#### 2.2.4.4 Assay of plasma triglycerides

The quantitative determination of plasma triglycerides was performed with a COBAS MIRA automated sample system using the triglycerides assay kit, with the C.f.a.s Calibrator and quality controls: Precinorm U and Precipath U (Roche Diagnostics, Australia). This was performed in a single assay with the intra-assay coefficient of variation < 1%.

#### 2.2.4.5 Assay of plasma $\alpha$ -amino nitrogen

Plasma  $\alpha$ -amino nitrogen concentration was determined using a previously reported method (Evans, Ffolliott-Powell *et al.* 1993). Plasma samples (100  $\mu$ l) were deproteinised by adding 800  $\mu$ l of 0.04M H<sub>2</sub>SO<sub>4</sub> and 100  $\mu$ l of 0.3M sodium tungstate and centrifuged for 15 minutes at 3000 rpm at 4°C. The supernatant was removed and pipetted in triplicate into 96 well ELISA plates. 24  $\mu$ l of sodium tetraborate (saturated, pH = 9.6) and 20  $\mu$ l 0.02 M  $\beta$ - naphthoquinone sulfonate (Sigma, Australia) were added to each well, and then plates were placed in a dry oven for 20 minutes at 80°C followed by 5 minutes in a -20°C freezer. 120  $\mu$ l of Milli-Q water, 36  $\mu$ l of acid formaldehyde (0.045M formaldehyde / 0.6M HCl) and 20  $\mu$ l 0.05M sodium thiosulphate were added per well and the plates were incubated at room temperature for 30 minutes. The plates were read with a dual wavelength ELISA plate reader at 490 nm and 660 nm. Six standards were prepared with concentrations ranging from 1mM to 0.1mM glycine. This was performed in a single assay with the intra-assay coefficient of variation < 8%.

#### *2.2.4.6 Assay of maternal plasma hormones*

Maternal plasma estradiol (Ultra-Sensitive Estradiol, DSL) and progesterone concentrations (Progesterone assay kit, DSL) were quantified with radioimmunoassay kits. These assays were performed by Mr Alan Gilmore (Repromed, Adelaide, Australia) and Mr Fred Amato (Department of Obstetrics and Gynaecology, University of Adelaide). This was performed in a single assay with the intra-assay coefficient of variation < 3%.

### **2.2.5 Measurement of maternal circulating IGF-I, IGF-II and total IGFBP**

#### *2.2.5.1 HPLC separation of plasma IGF-I and IGF-II from IGFBP*

This method was performed as previously described (Owens, Kind *et al.* 1994; Owens, Johnson *et al.* 1990). Plasma recovered (90  $\mu$ l) from mothers killed on day 35 of pregnancy (vehicle, n = 5; IGF-I, n = 5; IGF-II, n = 3) was acidified with 150  $\mu$ l of 4x mobile phase (800 mM glacial acetic acid, 200 nM trimethylamine, 400 mM hydrochloric acid and 0.05% (w/v) Tween-20, pH of 2.8, filtered using a 0.2 $\mu$ M GV Millipore filter and degassed) and 360  $\mu$ l of Milli-Q was added to make a final volume of 600  $\mu$ l. Samples were vortexed and then incubated at room temperature for 30 minutes.

To de-lipidate the sample, an equal volume (600  $\mu$ l) of freon (1,1,2-trichloro-1,2,2-trifluoroethane) was added and centrifuged for 10 minutes at 10,000 rpm. The upper, aqueous phase was removed and filtered by centrifugation for 3 minutes at 10,000 rpm through a microfilter containing a 0.45  $\mu$ m cellulose acetate filter membrane (Alltech Associates Inc., Australia). 300  $\mu$ l of filtered sample was loaded into HPLC injection vials, of which 150  $\mu$ l was injected onto a Protein-Pak 125 HPLC exclusion column (Waters/Millipore, Australia) using an automatic injector (ICI AS 2000, ICI Instruments, Australia). Samples were eluted at 1 ml/min in 1x mobile phase.

To establish where IGFs were eluting under these conditions, 36 fractions of 0.5 ml were collected (pooled guinea pig plasma) and each fraction was subjected to IGF-I and IGF-II RIA. The IGFBPs eluted between 0.5 and 2.5 minutes and free IGFs eluted between 3 and 5.5 minutes. On the basis of this elution profile, chromatogram fractions were collected at these times and subjected to RIA. The first fraction contained IGFBPs and the third fraction contained the purified IGFs.

### 2.2.5.2 RIA detection of IGFs

Recombinant human IGF-I and IGF-II (GroPep Pty. Ltd, Adelaide, Australia) were used as standards and for preparation of radiolabelled ligands. Ten serial 1:2 standards were prepared with concentrations ranging from 2000 pg/tube to 3.91 pg/tube. 100  $\mu$ l of each sample (HPLC fraction 3) was assayed in triplicate in polystyrene tubes, to which 200  $\mu$ l of RIA buffer was added (30mM Na<sub>2</sub>PO<sub>4</sub>, 0.02% (w/v) protamine sulphate, 10mM disodium EDTA, 0.05% (w/v) Tween-20 and containing 0.02% (w/v) NaH<sub>3</sub>, pH of 7.5). 100 $\mu$ l of 1x mobile phase was added to standard tubes and 60  $\mu$ l or 30  $\mu$ l of 0.4M Tris Base was added to each tube of the IGF-I or IGF-II assays, respectively, to neutralise to pH 7.4. 50  $\mu$ l of anti-IGF antibody and 50  $\mu$ l radio-iodinated human IGF (20,000 cpm) were also added to every tube. IGF-I was measured by RIA using rabbit anti-human IGF-I antiserum (MAC Ab 89/1, GroPep Pty, Ltd, Adelaide) at a final dilution of 1/60,000 and a monoclonal mouse anti-rat IGF-II antibody (kind gift from Dr K Nishikawa, Kanaza Medical University, Ishakawa, Japan) was used at a final concentration of 1/500 to measure IGF-II by RIA. Tubes were briefly vortexed and incubated for 18-20 hours at 4°C. As a measurement of total radioactivity (TC), three tubes that contained only radio-iodinated human-IGF were included in the assay. Additionally, three blank tubes were prepared containing RIA buffer and human-IGF. To serve as a positive control, a reference sample of known IGF concentration was run.

For the IGF-I assay, 50  $\mu$ l of sheep anti-rabbit IgG (Silenus, Australia), diluted 1/20 in RIA buffer and 10  $\mu$ l of rabbit IgG (Dako, Australia), also diluted 1/20 in RIA buffer, were added to each tube. For the IGF-II assay, 50  $\mu$ l of sheep anti-mouse IgG (Silenus, Australia), diluted 1/20 in RIA buffer and 10  $\mu$ l of mouse IgG (Dako, Australia), also diluted 1/20 in RIA buffer, were added to each tube. Tubes were vortexed and incubated at room temperature for 30 minutes. 1 ml of ice-cold polyethylene glycol (6% (w/v) polyethylene glycol 6000 in 0.9% (w/v) NaCl) was added to all tubes except TC, vortexed and then centrifuged for 20 minutes at 4000 rpm, at 4°C (J-6B Beckman Instruments, USA). The supernatant was carefully aspirated and the pellet was counted for <sup>125</sup>I using a gamma scintillation counter (1261 Multigamma, LKB Pharmacia and Wallace, Sweden).

Cross-reactivity of IGF-II in the IGF-I RIA was less than 1% (Francis, McNeil *et al.* 1989; Francis, Owens *et al.* 1989) and that of IGF-I in the IGF-II RIA was less than 2.5% (Carr, Owens *et al.* 1995). Both IGF-I and IGF-II amino acid sequences are remarkably conserved across species. Guinea pig IGF-I and IGF-II have previously been shown to have 100% amino acid sequence identity to those of human (Bell, Stempien *et al.* 1990; Levinovitz, Norstedt *et al.* 1992), while guinea pig IGF-II has only one amino acid different to that of the rat (Ekstrom, Backlin *et al.* 1993). We have previously reported that the recoveries

of IGF-I and IGF-II are >95% for these assays (Sohlstrom, Katsman *et al.* 1998a). The minimal detectable concentrations of IGF-I and IGF-II were 6.64 ng/ml and 9.48 ng/ml, respectively. The samples were analysed in a single RIA, where the mean intra-assay coefficients of variation were 3.7% and 5.6% for IGF-I and IGF-II RIAs, respectively.

### *2.2.5.3 Measurement of IGFBP fraction*

The total IGFBP binding capacity in the maternal circulation was indirectly measured as the interference of the IGFBPs in fraction 1 in the IGF-I RIA (refer to 2.2.5.2), which has been previously described (Francis, McNeil *et al.* 1989; Francis, Owens *et al.* 1989; Owens, Johnson *et al.* 1990). A lower cpm value is indicative of increased unbound IGF present in the plasma, therefore the higher the cpm, the greater amount of IGFBP is present in the sample to bind to the radio-iodinated IGF-I. The ratio of IGFs to IGFBPs provided an index of IGF bioavailability in the maternal circulation.

### *2.2.6 Western ligand blots*

Individual IGF binding proteins in samples of pooled maternal plasma recovered from vehicle, IGF-I and IGF-II treated animals killed on day 35 or day 62 of pregnancy, were identified by Western ligand blotting. Samples were prepared by incubating 20  $\mu$ l of plasma with 130  $\mu$ l of Milli-Q water and 50  $\mu$ l of 4x sodium dodecyl sulfate (SDS) at 65°C for 20 minutes. Prepared samples (20  $\mu$ l, loaded with a 100  $\mu$ l Hamilton syringe) were then subjected to non-reducing discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4% stacking gel and a 12% polyacrylamide separating gel, for 90 minutes at 20 mA (500 V) followed by overnight at 8 mA. Molecular masses of IGFBP bands were calculated from <sup>14</sup>C-labeled "rainbow" molecular-weight markers (GE Healthcare, formerly Amersham Biosciences, Australia), which were incubated with 10  $\mu$ l Milli-Q water and 20  $\mu$ l 4x SDS, at 65°C for 20 minutes, prior to separation by gel electrophoresis.

Separated proteins were then transferred onto a nitrocellulose membrane at 250 mA for 1 hour (2 gels), which were dried overnight. The following day, nitrocellulose membranes were washed in 1% Triton (1% Triton X in 2M NaCl / 0.5M Tris base buffer) for a maximum of 30 minutes and blocked with 1% bovine serum albumin (BSA, RIA grade, Sigma, Australia) (1% BSA in 2M NaCl / 0.5M Tris base buffer), for 90 minutes. Membranes were washed in 0.1% Tween (1% Tween 20 in 2M NaCl / 0.5M Tris based buffer) for 10 minutes at room temperature with shaking. Nitrocellulose membranes were then probed with radio-iodinated human IGF-II (10,000,000 cpm in 1% BSA used for blocking membranes) for 2 hours and 30 minutes at room temperature with shaking. Nitrocellulose membranes were then washed in three



changes of fresh 0.1% Tween, for 30 minutes each. Nitrocellulose membranes were dried overnight and then exposed to X-ray film for 2-4 weeks at -80°C, before developing.

### *2.2.7 Placental Histology using Masson's Trichrome Stain*

#### *2.2.7.1 Procedure*

Placental tissue fixed overnight in 4% PFA (refer to 2.2.3) was washed four times in PBS (Dulbeccos Mg<sup>2+</sup> Ca<sup>2+</sup> free) over three days and stored in 70% ethanol at 4°C. Tissues were dehydrated through a series of ethanol washes of increasing concentration and de-waxed in two changes of xylene before immersion into paraffin wax at 55°C under a vacuum. Tissues were embedded in paraffin and then 5 µm mid-sagittal sections were cut using a Leica rotary microtome (Leica Microsystems, USA) and placed on silane-coated slides (3-aminopropyltriethylsilane, Sigma, Australia). For staining, mid-sagittal slices of paraffin embedded placentae were dewaxed using two Safsolvent (Ajax APS Chemicals, Australia) treatments of 5 minutes each, rehydrated gradually through ethanol washes to water and then placed in PBS. Sections were then stained with Masson's Trichrome (Drury and Wallington 1980). From each dam, 1-3 placentae were randomly selected for histological assessment.

Briefly, sections were stained in Weigert's Haematoxylin for 15 minutes. Sections were then washed in running tap water for 10 minutes, differentiated in 0.5% hydrochloric acid in 70% ethanol and then rinsed in tap water. Sections were then stained in red cytoplasmic stain (1% ponceau de xylydine, 1% acid fuschin, in 1% acetic acid) for 2 minutes and rinsed in two washes of Milli-Q water. This stain was then differentiated in 1% of phosphomolybdic acid (Sigma, Australia) for 1 minute and rinsed in Milli-Q. Sections were then stained with methyl blue (1% methyl blue in 1% acetic acid) for 3 minutes and washed twice in 1% (v/v) acetic acid in Milli-Q. Using Whatman filter paper, sections were blotted dry and then dehydrated through three changes of absolute ethanol and cleared in three changes of Safsolvent (Ajax Fine Chemicals, Australia) before mounting in DPX (Ajax Chemicals, Australia) with a glass coverslip.

#### *2.2.7.2 Video Image Analysis*

The mid-sagittal cross-sectional areas of the placental interlobium (germinative region) and labyrinth (exchange region) were measured in complete mid-sagittal sections using an Olympus BH-2 microscope with 2x objective and 3.3x ocular lenses and video image analysis software (Video Pro, Leading Edge, Adelaide) by the researcher blinded to treatment. The video image was calibrated to micrometers using a hemocytometer, allowing direct measurements to be made (Neubauer Bright Line,

Germany). The proportion (%) of each region in the placenta was then estimated by dividing the cross-sectional area of that region by the total mid-sagittal cross-sectional area of the placenta. An estimate of the volume of these regions was then calculated by multiplying their proportion by total placental weight.

### ***2.2.8 Structure of the placental exchange region (labyrinth)***

#### *2.2.8.1 Procedure*

To distinguish cell types within the placental labyrinth, mid-sagittal sections of placenta were double-labelled with mouse antibodies to human vimentin (3B4, Dako, Denmark) and human pan cytokeratin (C2562, Sigma, USA) to identify fetal capillaries and trophoblast, respectively, and then stained with eosin to aid the identification of maternal blood spaces. This employed a triple layer technique for each antibody, performed sequentially.

Sections were deparaffinized and brought to PBS as previously described (refer to 2.2.7.1). Deparaffinised sections underwent antigen retrieval by incubation at 37°C for 15 minutes in 0.03% protease (Sigma, USA). Following three 5 minute washes in PBS, endogenous peroxidase activity was quenched by incubating sections with 3% hydrogen peroxide in water for 30 minutes at room temperature. Sections were then washed in three changes of PBS for 5 minutes each and blocked for non-specific binding with serum-free protein block (Dako, Denmark) for 10 minutes. Without washing off the protein block, 3B4 antibody diluted 1:50 with 10% normal guinea pig serum and 1% BSA (made fresh w/v in PBS) was applied first and incubated overnight (~18 hours) in a humidified chamber at room temperature.

The following day, sections were washed as above, and biotinylated goat anti-mouse IgG secondary antibody (Dako, Denmark) was applied for 30 minutes, followed by washing to remove unbound secondary antibody. Streptavidin conjugated to horseradish-peroxidase (Rockland Immunochemicals, USA) was applied for 40 minutes, then sections were washed as above. 3B4 binding was visualized by incubating with 3,3-diaminobenzidine peroxidase substrate and urea hydrogen peroxide tablets dissolved in Milli-Q (Sigma Fast™; Sigma, Australia) with 2% (w/v) ammonium nickel (II) sulfate (Sigma, USA) to form a black precipitate.

The process was then repeated for the second primary antibody (C2562) diluted 1:50 with PBS, 10% normal guinea pig serum, and 1% BSA (made w/v in PBS), but nickel was omitted from the chromogen, leaving a brown precipitate. Negative controls used irrelevant mouse IgG in place of the primary

antibodies or the primary antibody diluent on its own. Samples were then counterstained with filtered Gill's haematoxylin (Sigma, Australia) for 40 seconds, differentiated in 5-10 seconds immersion in 0.5% (v/v) ammonia water, 5 seconds in 0.1% (v/v) hydrochloric acid and 5-10 second immersion in 0.5% (v/v) ammonia water again. Sections were briefly stained with eosin before they were dehydrated, cleared and mounted in DPX (Ajax Fine Chemicals, Australia) with a glass coverslip.

#### *2.2.8.2 Morphometric Analysis*

The mid-sagittal placental labyrinth was then morphometrically analysed, as previously described (Roberts, Sohlstrom *et al.* 2001a). Briefly, the proportions (volume density) and volumes of the labyrinthine placental components were quantitated by point counting on ten non-overlapping fields on a monitor with a L-36 Merz grid to quantify the proportion, with random systematic sampling using an Olympus BH-2 microscope with 20x objective and 3.3x ocular lenses. The volume of each component was estimated by multiplying the volume density by the weight of the placental labyrinth. An estimate of the surface area per gram of placental labyrinth was quantitated using intercept counting and the total surface area of the syncytiotrophoblast for exchange and arithmetic mean trophoblast thickness (the layer through which substrate exchange occurs) were calculated (as previously described (Weibel 1979)). These analyses were performed by the researcher blinded to treatment.

#### *2.2.9 Protein localisation of IGF receptors in the placenta on day 35 of pregnancy*

To determine that the placenta expressed IGF1R and IGF2R at the time of treatment, we localised them in placental sections from the cohort of guinea pigs that were killed on day 35 of pregnancy in which circulating IGFs had been quantified. Mid-sagittal slices of placentae were immuno-labelled with rabbit antibodies raised against human IGF1R (N-20, diluted 1:20, Santa Cruz Biotechnology) and IGF2R (a kind gift from Dr Carolyn Scott, Kolling Institute of Medical Research, Sydney, Australia, diluted 1:100). This employed a triple layer technique for each antibody performed on serial placental sections, as described above (refer to section 2.2.8.1), but using biotinylated goat anti-rabbit IgG secondary antibody (Dako, Denmark). Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

### *2.2.10 Measurement of plasma and tissue MG and AIB content*

#### *2.2.10.1 Preparation of quench standards*

The DPM of  $^3\text{H}$  and  $^{14}\text{C}$  in samples was calculated from CPM and efficiency by dual isotope  $\beta$ -scintillation counting using quenched standards prepared from tritiated water ( $^3\text{H}_2\text{O}$ ) and  $^{14}\text{C}$ -AIB, respectively. Ten quench standards were prepared using 1 ml of Milli-Q water, 30  $\mu\text{l}$  of glacial acetic acid, 14 ml of aqueous scintillation fluid (Beckman, USA), 50  $\mu\text{l}$  of  $^3\text{H}_2\text{O}$  or  $^{14}\text{C}$ -AIB (55,000 DPM) and varying volumes (0, 0, 10, 20, 40, 60, 80, 100, 120 and 120  $\mu\text{l}$ ) of carbon tetrachloride in 20 ml plastic scintillation vials. These were shaken, covered with foil and counted the following day after shaking once more by dual isotope  $\beta$ -scintillation counting (LS 6500 Beckman, USA) at 0-400 and 400-670 MeV for  $^3\text{H}$  and  $^{14}\text{C}$ , respectively.

#### *2.2.10.2 Plasma MG and AIB content determination*

For determination of plasma MG and AIB concentrations, 50  $\mu\text{l}$  of plasma was deproteinised with 100  $\mu\text{l}$  of 0.3 N,  $\text{Ba}(\text{OH})_2$  (Sigma Diagnostics, St Louis) and 100  $\mu\text{l}$  of 0.3 N,  $\text{ZnSO}_4$  (Sigma Diagnostics, St Louis) at  $4^\circ\text{C}$ . After centrifugation at 4000 rpm at  $4^\circ\text{C}$  for 15 minutes, 80  $\mu\text{l}$  of deproteinised supernatant was mixed with 0.3 ml of water, 30  $\mu\text{l}$  of glacial acetic acid and 14 ml scintillant (Ready Safe, Beckman Coulter, USA), before counting to measure  $^3\text{H}$  and  $^{14}\text{C}$  dpm by dual isotope  $\beta$ -scintillation counting (LS 6500 Beckman Coulter, USA) with quenched standards to indicate MG and AIB content, respectively.

#### *2.2.10.3 Tissue MG and AIB content determination*

For determination of fetal and maternal tissue MG and AIB concentrations, tissues (~100 mg) were solubilized with 0.7 ml 1M NaOH for 85 minutes at  $65^\circ\text{C}$  and then mixed with 2.1 ml 6% perchloric acid to precipitate protein for 20 minutes at  $4^\circ\text{C}$ . After centrifugation at 4000 rpm at  $4^\circ\text{C}$  for 15 minutes, 1 ml of deproteinised supernatant was mixed with 30  $\mu\text{l}$  of glacial acetic acid and 14 ml scintillant (Ready Safe, Beckman Coulter, USA) and dual isotope  $\beta$ -scintillation counting (LS 6500 Beckman Coulter, USA) with quenched standards (as described for plasma MG and AIB determination).

#### *2.2.10.4 DPM Calculations*

The background DPM was then subtracted from samples, before sample DPM per gram or ml or total was adjusted to the same dose of isotope administered to the mother. DPM were represented as DPM / ml for plasma samples. For tissues, DPM were represented as DPM / g or total tissue uptake by multiplying the DPM / g by the weight of the tissue. Perirenal, retroperitoneal and interscapular fat counts were combined to provide a measure of fat uptake; gastrocnemius and triceps counts were

combined to provide a measure of muscle uptake; heart, spleen, kidney, liver and lung counts were combined to provide a measure of visceral tissue uptake; and all tissues assessed for analogue studies were combined to represent total tissue uptake. To estimate the transfer capacity of the placenta, the total fetal tissue counts were divided by placental weight.

### *2.2.11 Statistics*

To assess differences in fetal weight distribution between treatments, chi squared tests were performed using Microsoft Excel. All other data were analysed using SPSS version 13 (SPSS, Chicago). To assess differences in maternal weight gain repeated measures ANOVA with Bonferroni Post Hoc Tests were performed. To assess differences in maternal body composition, general linear model univariate ANOVA with Bonferroni Post Hoc Tests were performed. To assess differences in fetal and placental parameters, linear mixed model repeated measures ANOVA with Sidak Post Hoc Tests were performed with the mother as a subject and the fetus or placenta as the repeated measure. The number of viable pups per litter were used as a covariate when required. Using Pearson's two-Tailed Bivariate Correlational analyses, associations between MG and AIB uptake and placental structure and maternal circulating hormone concentrations were performed. Data are expressed as mean  $\pm$  SEM or estimated marginal mean  $\pm$  SEM as required. Data were considered statistically significant when  $P < 0.05$ .

*Chapter 3 : Effect and interaction of  
oxygen concentration and exogenous  
IGF-II in early human placenta*

### 3.1 Introduction

The placenta is essential for development of the fetus and the success of pregnancy. It forms the functional interface between the developing fetus and mother and plays roles in nutrient, waste, and gas exchange, endocrine, immune and metabolic functions (Cross, Werb *et al.* 1994). The human placenta is composed of tree-like structures, called chorionic villi that consist of fetal capillaries in a mesenchymal core, which is surrounded by a monolayer of cytotrophoblast, which either fuse to form the overlying multinucleate syncytiotrophoblast, or differentiate into extravillous cytotrophoblast (EVT) that form cell columns at villous tips and invade the maternal uterine tissue and spiral arteries. Invasion of the maternal spiral arteries by the EVT results in remodelling and transformation of vessels into dilated capillaries unresponsive to maternal vasocontrol that permit uninterrupted high conductance maternal blood flow required for fetal growth (Brosens, Robertson *et al.* 1967; Kam, Gardner *et al.* 1999; Pijnenborg, Bland *et al.* 1983). As insufficient invasion and remodelling of maternal vasculature is associated with pregnancy complications, such as preeclampsia, intrauterine growth restriction and stillbirth (Domisse and Tiltman 1992; Khong, De Wolf *et al.* 1986; Khong, Liddell *et al.* 1987; Kim, Bujold *et al.* 2003; Robertson, Brosens *et al.* 1967) it is imperative that we understand the factors regulating trophoblast function to better understand these pathologies.

It is well established that in humans oxygen concentration/availability at the fetomaternal interface is a major influence on cytotrophoblast function during early placentation (Caniggia and Winter 2002; Genbacev, Zhou *et al.* 1997). Indeed, direct connections between the uterine spiral arteries and the intervillous space (IVS) of the placenta cannot be observed before the ninth week of gestation (Hamilton and Boyd 1960; Harris and Ramsey 1966). Before that stage, the distal segments of the arteries are occluded by aggregates of invading EVTs (Burton, Jauniaux *et al.* 1999; Hamilton and Boyd 1960; Harris and Ramsey 1966; Hustin and Schaaps 1987), which expose villous and extravillous trophoblast to a relatively hypoxic/low oxygen environment in early pregnancy (~18 mmHg, ~2-3% O<sub>2</sub>) that is considerably lower than that detected in the endometrium (~40 mmHg, 5-6% O<sub>2</sub>) (Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992). During this period, the placenta grows at a faster rate than the embryo. This low-oxygen environment in early pregnancy is also essential for normal embryonic and placental development as studies have shown that early onset of maternal blood flow into the IVS is associated with early pregnancy loss (Hustin, Jauniaux *et al.* 1990; Jauniaux, Greenwold *et al.* 2003; Jauniaux, Zaidi *et al.* 1994).

Near the end of the first trimester, endovascular trophoblastic plugs are progressively dislodged, exposing the developing placenta to maternal blood flow from ~10 weeks of gestation (Jaffe and Woods 1993; Jauniaux, Jurkovic *et al.* 1992). Indeed, placental oxygen tension in the IVS increases nearly 3-fold (>60mmHg, 8-9%) after 10-12 weeks gestation (Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992) exposing trophoblasts to increased oxygen tension at this time and indicating that maternal blood is flowing into the intervillous spaces (Jaffe and Woods 1993; Jauniaux, Jurkovic *et al.* 1992; Jauniaux, Watson *et al.* 2000).

The exact mechanism by which trophoblasts sense oxygen tension is currently unclear however, several potential pathways have been identified. The most characterised mechanism in trophoblast involves the formation of the hypoxia-inducible factor (HIF) protein complex. HIF is a heterodimeric basic helix-loop-helix PAS transcription factor composed of an  $\alpha$  and a  $\beta$  subunit. Both HIF subunits are constitutively expressed, although under normoxic conditions the  $\alpha$  subunit is rapidly hydroxylated and subsequently targeted for proteolytic degradation (Ivan, Kondo *et al.* 2001; Jaakkola, Mole *et al.* 2001; Masson, Willam *et al.* 2001; Maxwell, Wiesener *et al.* 1999; Yu, White *et al.* 2001). Under hypoxic conditions, the absence of oxygen prevents hydroxylation and therefore degradation of the  $\alpha$  subunit, which can then translocate from the cytoplasm to the nucleus and dimerise with the  $\beta$  subunit (Huang and Bunn 2003; Wang, Jiang *et al.* 1995a; Wang, Jiang *et al.* 1995b). This forms the active HIF complex which binds to the short DNA consensus sequence, the hypoxia response element (HRE, 5' -RCGTG-3') in the promoter region of various genes and induces their transcription (Zagorska and Dulak 2004). Among the variety of molecules whose expression is induced under low oxygen are those involved in glycolytic pathways and vasculogenesis. Studies have also demonstrated that the gene encoding insulin-like growth factor-II (IGF-II), which is important in fetal and placental growth, is also induced in some cell types by hypoxic exposure (Feldser, Agani *et al.* 1999; Kim, Bae *et al.* 1998; Steinbrech, Mehrara *et al.* 2000; Tucci, Nygard *et al.* 1998).

IGF-II is expressed by the fetus and placenta during pregnancy and is particularly abundant in the EVT invading the maternal endometrium and spiral arteries in women (Han, Bassett *et al.* 1996; Ohlsson, Holmgren *et al.* 1989; Ohlsson, Larsson *et al.* 1989) and other species (Han and Carter 2000; Redline, Chernicky *et al.* 1993; Zhou and Bondy 1992). This spatial expression of IGF-II suggests a role for this protein in regulating trophoblast function. Indeed, IGF-II stimulates glucose and amino acid uptake (Kniss, Shubert *et al.* 1994), proliferation (Ohlsson, Holmgren *et al.* 1989), migration (Irving and Lala 1995; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001) and invasion (Hamilton,



Lysiak *et al.* 1998) of cultured human trophoblast cell lines. Studies performed in genetically modified mice either completely deficient in *Igf2* (Baker, Liu *et al.* 1993; DeChiara, Efstratiadis *et al.* 1990; Lopez, Dikkes *et al.* 1996) or with impaired placental *Igf2* gene expression (Constancia, Hemberger *et al.* 2002) display reduced placental development with subsequent perturbations in fetal growth.

IGF-II can bind to both types of IGF receptor, IGF1R and IGF2R, as well as the insulin receptor (InsR). The biological activities of IGF-II are primarily thought to be mediated through the IGF1R, which is a disulfide-linked heterotetrameric ( $\alpha_2\beta_2$ ) transmembrane glycoprotein with an extracellular ligand-binding  $\alpha$ -subunit and intracellular  $\beta$ -subunit with tyrosine kinase activity (Nissley, Haskell *et al.* 1985). The InsR plays a role in embryonic development (Louvi, Accili *et al.* 1997) but its involvement in IGF-II effects on placental development is unclear. IGF2R is a single transmembrane receptor identical to the cation-independent mannose-6-phosphate receptor, consisting of a large extracellular domain and a small cytoplasmic tail that lacks tyrosine kinase activity (Ludwig, Le Borgne *et al.* 1995). IGF2R is thought to primarily function as a clearance receptor for IGF-II and thereby modulates extracytoplasmic concentrations of IGF-II (Ludwig, Eggenschwiler *et al.* 1996; Ludwig, Le Borgne *et al.* 1995). However, recently, a role for IGF2R in mediating IGF-II effects on trophoblast invasion has been reported (Hamilton, Lysiak *et al.* 1998). In addition, studies in human monocytes have revealed the membrane bound IGF2R is capable of activating latent TGF- $\beta$ 1 (LTGF- $\beta$ 1) (Godar, Horejsi *et al.* 1999), an inhibitor of trophoblast invasion (Graham and Lala 1991; Graham and Lala 1992) via an interaction that involves the urokinase plasminogen activator receptor (uPAR).

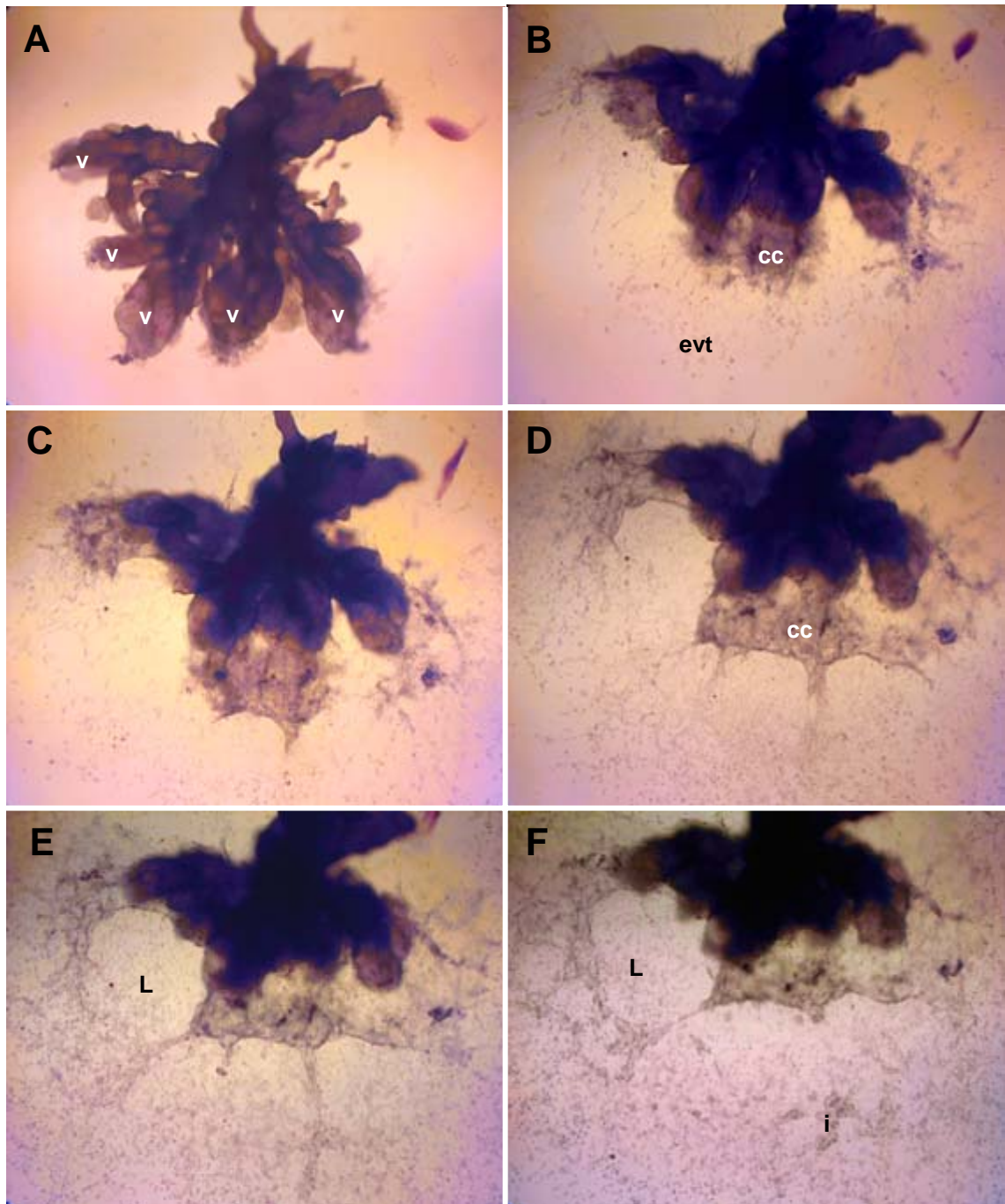
Therefore, we have proposed that interactions between oxygen concentration, IGF-II, and TGF- $\beta$ 1 activation may be key to the regulation of placental development. The aims of this investigation were to examine the effect and interaction of oxygen concentration and exogenous IGF-II treatment on first trimester human placental growth, gene expression and TGF- $\beta$  activation *in vitro*. To achieve this we employed a first trimester placental villous explant system, involving the culture of placental villi in serum-free media, on an artificial extracellular matrix (Matrigel) and exposing the explants to 1%, 5% or 20% oxygen with or without exogenous IGF-II (6 treatments).

## 3.2 Results

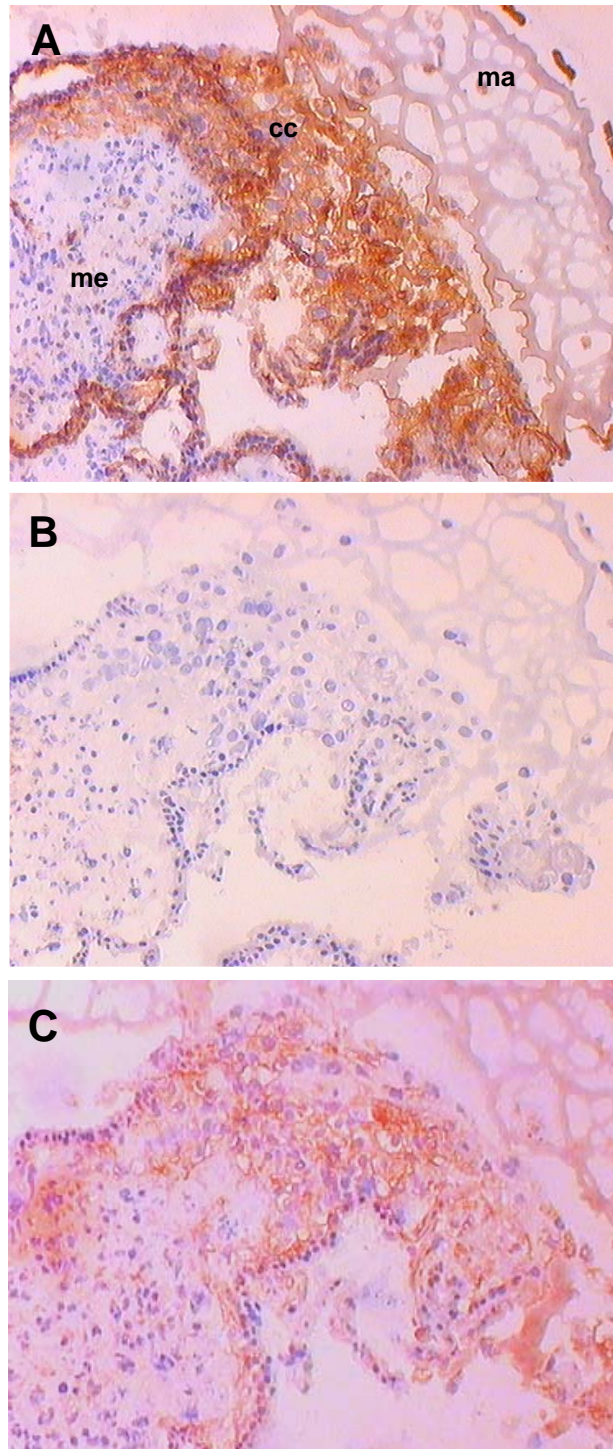
### 3.2.1.1 First trimester villous explant growth

Villous explants were established from first trimester human placenta obtained from women undergoing elective termination of pregnancy at 7-12 weeks of gestation and morphological integrity and growth over the 6 days in culture were assessed in photomicrographs taken daily. EVT outgrowth from the distal end of the villous tips and their migration into the surrounding extracellular matrix (Matrigel) were observed over the culture period (Figure 3.1). Following only two days in culture, explants developed dense, three-dimensional outgrowths (cell columns) at the distal tips of villi (Figure 3.1b). Cells dissociated from the cell column (EVTs) and migrated away from the villous tips radially as individual cells (evc, Figure 3.1b). Cell columns aggregated with adjacent cell columns at 4 days of culture (cc, Figure 3.1d) and extensive migration of EVT's lead to the formation of looped outgrowth (L, Figure 3.1e) and/or dense balloon-like structures (not shown). By day 6 in culture it appeared that some cells that originally comprised cell columns, had detached and migrated away from the villous tip in clusters of cells deemed cell islands (i, Figure 3.1e).

To confirm that outgrowing/migrating cells were trophoblasts, explants were frozen and immunohistochemically labelled for cytokeratin-7 and also for the expression of IGF-II (Figure 3.2). Antibodies against cytokeratins are commonly employed to identify cells of epithelial origin, while cytokeratin-7 identifies trophoblast (Blaschitz, Weiss *et al.* 2000; King, Thomas *et al.* 2000). In these explants, there being no attached decidua, the only epithelial cells will be trophoblasts. Cells that formed the outgrowths (cell columns) at the tips of villi characteristically displayed a vacuolated appearance with large nuclei, which was most obvious in the negative controls (Figure 3.2b). The STB, CTB and cells constituting the extravillous cell columns labelled for cytokeratin-7, whereas the villous mesenchyme was negative (Figure 3.2a). Matrigel, the ECM substrate used in the villous explant cultures, was also seen to non-specifically label (Figure 3.2a). IGF-II protein was localised to CTB and less abundantly to the STB and mesenchyme of villous explants (Figure 3.2c)



**Figure 3.1** Daily growth of human placental villous explants. First trimester (7 weeks gestation) human placental villous explants after 1 day (a), 2 days (b), 3 days (c), 4 days (d), 5 days (e) and 6 days (f) in culture in serum-free media in 1% oxygen. Note the formation of cell columns (cc) at the tips of villi (v) and the detachment and migration away from villous tips of individual extravillous cells (evt) after only 2 days in culture (b). Cell columns began to aggregate with adjacent cell columns (cc) following 4 days of culture (d) and after 5 days in culture (e), extravillous cell migration was extensive and exhibited a loop-like appearance (L). Some cells that originally comprised cell columns, appeared to have detached and migrated away in clusters forming extravillous cell islands (i) after 6 days in culture (f). Photomicrographs taken with an inverted microscope with 4x objective.



**Figure 3.2 Cytokeratin-7 and IGF-II immunolabelling of villous explants.** Trophoblast was identified in fresh-frozen first trimester (7 weeks gestation) human placental villous explants that had been cultured for 6 days using immunohistochemical labelling and counterstained with haematoxylin. Cells that were positive for cytokeratin-7 (a) or IGF-II (c) stained brown and negative control section shown (b). Cell column (cc), Matrigel (ma), mesenchyme (me). Photomicrographs taken with a brightfield microscope under 10x objective.

### *3.2.1.2 The effect of gestational age on villous explant outgrowth in response to oxygen concentration*

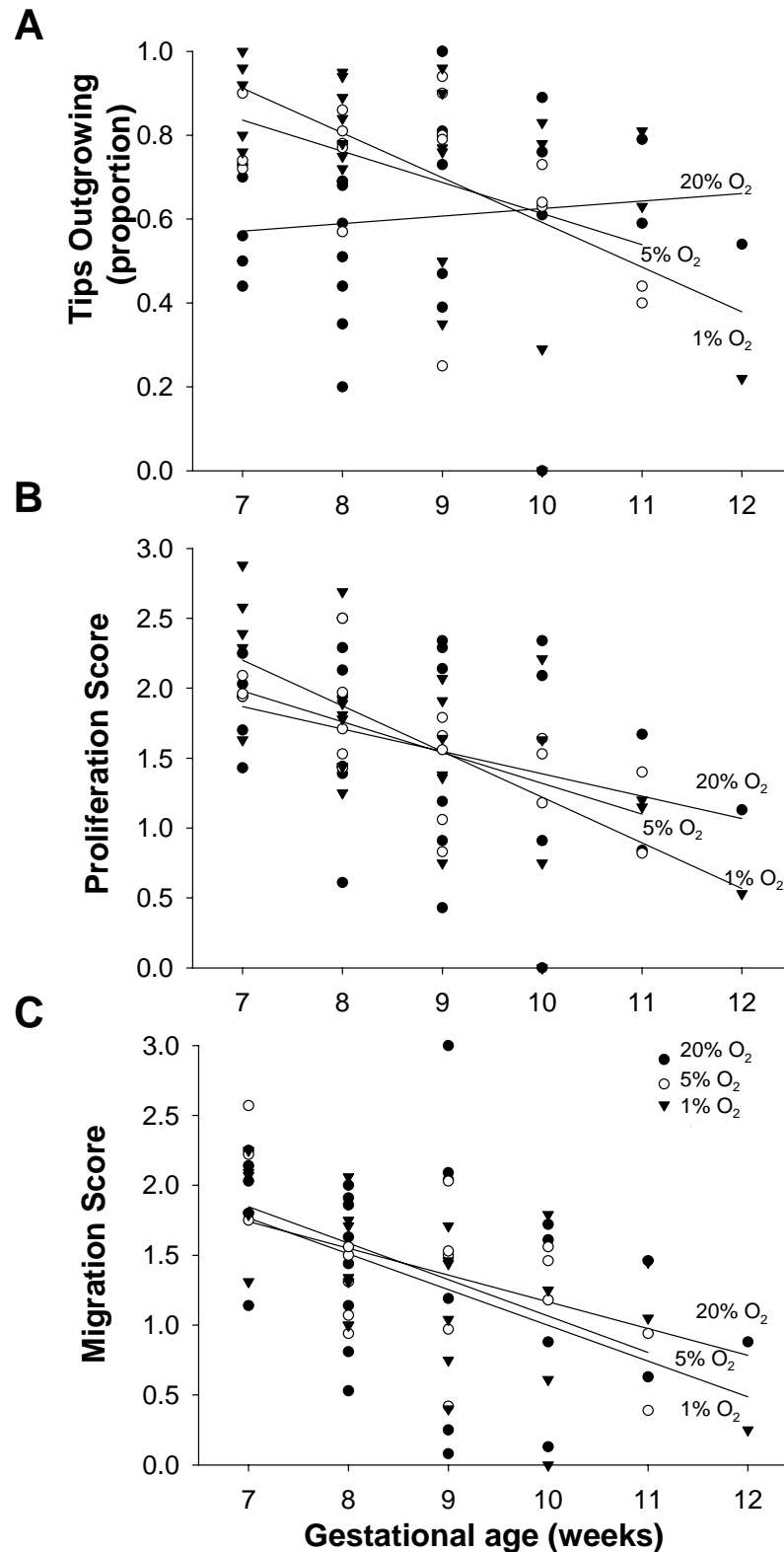
To determine whether there was a differential response of first trimester placental villous explants to oxygen concentration with gestational age, villous explants of 7-12 weeks gestation were cultured in 20%, 5% and 1% oxygen atmospheres. There was no effect of gestational age on the proportion of tips outgrowing in explants cultured in 20% oxygen. However, there was a negative correlation between gestational age and proportion of villous tips outgrowing in those exposed to 5% and 1% oxygen atmospheres ( $r = -0.50$ ,  $P = 0.034$  and  $r = -0.58$ ,  $P = 0.003$ , respectively) (Figure 3.3a). Regardless of oxygen concentration, gestational age of the villous explants was negatively correlated with villous tip proliferation score ( $r = -0.51$ ,  $P < 0.001$ ) and migration score ( $r = -0.49$ ,  $P < 0.001$ ) (Figure 3.3b and c).

To determine whether the gestational age of the placenta affected the responsiveness of explants to oxygen concentration, the percent difference of outgrowth for individual placental explants grown in 20% oxygen was compared with 5% and 1% explants (Figure 3.4). There was significant reduction in the proportion of tips outgrowing and extent of proliferation and migration from those tips in placentas from 11 and 12 weeks of gestation in those cultured in 5% oxygen compared to 20% oxygen controls (Figure 3.4a-c). In response to 1% oxygen atmospheres, there was significant reduction in the proportion of tips outgrowing and extent of proliferation in placenta at 10 weeks and migration from those tips at 9 weeks of gestation (Figure 3.4 d-f). Given the differential response of placenta at different gestational ages on explant outgrowth, data for 7-8 weeks gestation were grouped together as were data from 9 to 12 weeks gestation.

### *3.2.1.3 Effect and interaction of oxygen and exogenous IGF-II on villous outgrowth at 7-8 weeks of gestation*

To determine the effect and interaction of oxygen concentration and IGF-II on the formation of extravillous cytotrophoblast outgrowths, human first trimester villous explants obtained at 7-8 weeks of gestation were cultured under 20%, 5% and 1% oxygen atmospheres with and without the addition of 17nmol IGF-II. The morphologies of the resultant outgrowth patterns were compared (Figure 3.5).

Culture of explants under low oxygen atmospheres increased the proportion of tips outgrowing by 42% under 5% oxygen and by 58% under 1% oxygen compared with those cultured in 20% oxygen (mean  $\pm$  SEM; 20% oxygen:  $0.53 \pm 0.03$ , 5% oxygen:  $0.75 \pm 0.04$ , 1% oxygen:  $0.83 \pm 0.03$ , both  $P < 0.001$ , Figure 3.5a). Exogenous IGF-II enhanced cell column formation by 37% in 20% oxygen explants



**Figure 3.3 Association of gestational age and villous explant outgrowth under different oxygen concentrations.** The proportion of villous tips bearing cell columns per explant (a) and the extent of proliferation (b) and migration (c) from those tips in response to 20%, 5% and 1% oxygen atmospheres. Each data point represents mean value of 8 explants per placenta (closed circles, 20% oxygen; open circles, 5% oxygen; closed triangles, 1% oxygen). Number of placentas per oxygen concentration = 23, thus giving a total 69 placentas. Regression lines for each oxygen concentration are displayed on the graph. Refer to text for *r* and *P* values.

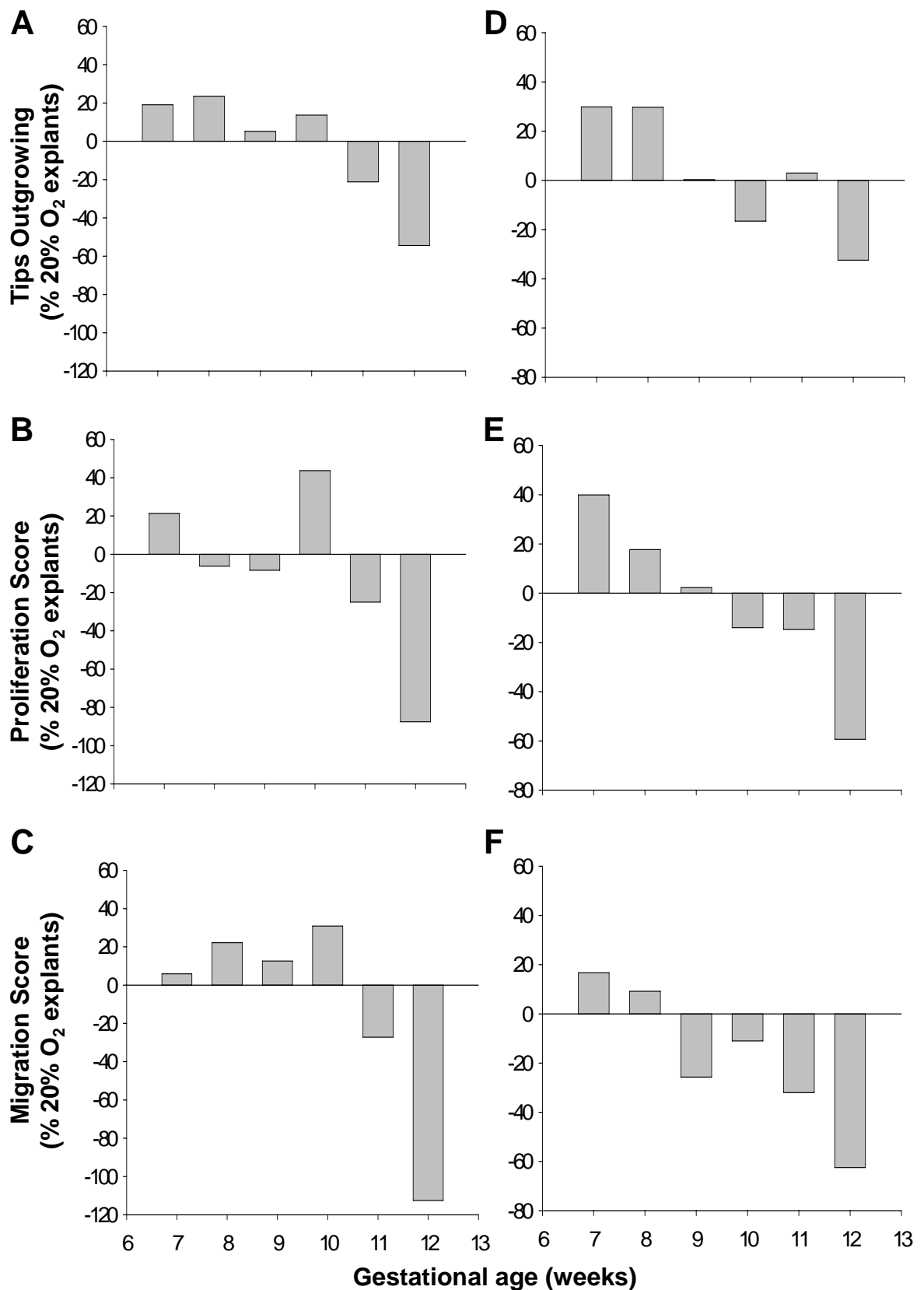


Figure 3.4 Effect of gestational age with the growth response of villous explants to oxygen concentration. Villous explant outgrowth in response to 5% oxygen (a-c) and 1% oxygen (d-f), across gestational age was expressed as a percentage of the explants cultured in 20% oxygen atmospheres (as indicated by zero line). Growth responses were indicated by proportion of villous tips bearing cell columns per explant (a, d) and the extent of proliferation (b, e) and migration (c, f) from those tips. Figures represent the mean differential response within each placenta  $\pm$  SEM.

(mean  $\pm$  SEM =  $0.72 \pm 0.03$  versus  $0.53 \pm 0.03$ , respectively,  $P < 0.001$ ), whereas it did not affect tip outgrowth in explants cultured in 1% or 5% oxygen. The outgrowth seen in 20% oxygen explants with exogenous IGF-II was similar to that seen in 1% and 5% oxygen explants.

By arbitrarily assessing the appearance of the EVT's as being round, dendriform or a mixture, dendriform being a phenotype attributed to migratory cells, it was found that there was a higher proportion of EVT's displaying a dendriform migratory phenotype in 5% and 1% oxygen explants compared with 20% oxygen ( $P < 0.047$  and  $P < 0.001$ , Figure 3.5b). Addition of exogenous IGF-II to each of the explants had no effect on EVT appearance (Figure 3.5b). There was no effect of oxygen concentration or exogenous IGF-II on the extent of villous tip proliferation or migration (Figure 3.5c, d).

#### *3.2.1.4 Effect and interaction of oxygen and exogenous IGF-II on villous outgrowth at 9-13 weeks of gestation*

To determine the effect and interaction of oxygen concentration and IGF-II on the formation of extravillous cytotrophoblast outgrowths, human first trimester villous explants obtained at 9-13 weeks of gestation were cultured in 20%, 5% and 1% oxygen atmospheres with and without the addition of 17nmol IGF-II, and the morphology of the resultant outgrowth patterns were compared.

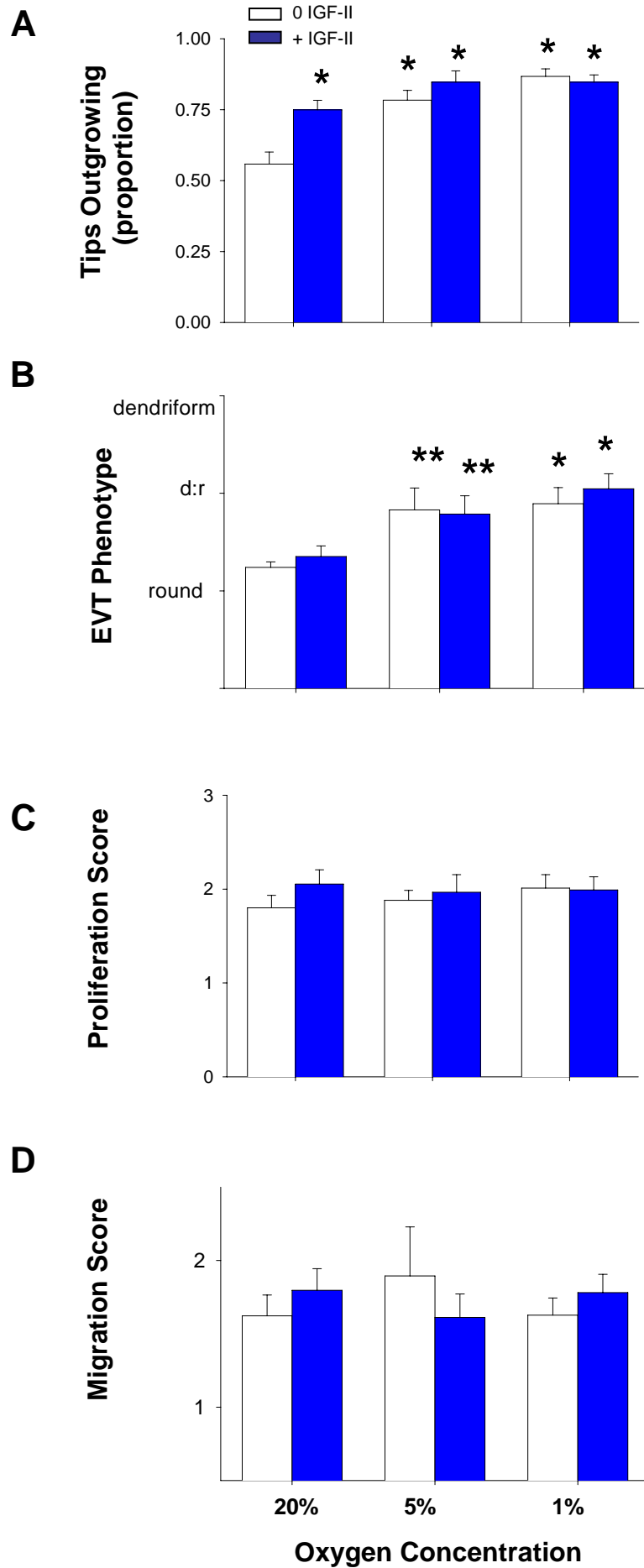
There was no effect of oxygen concentration on any of the four villous outgrowth parameters assessed (Figure 3.6). In explants exposed to 20% oxygen, exogenous IGF-II increased the proportion of villous tips bearing cell columns by 22% ( $P = 0.047$ ) and the proliferation (+31%,  $P = 0.04$ ) and migration (+33%,  $P = 0.035$ ) of trophoblast from those tips, without altering the phenotype of the EVT's (Figure 3.6a, c, d).

#### *3.2.1.5 Effect of gestational age on villous explant outgrowth in response to IGF-II treatment*

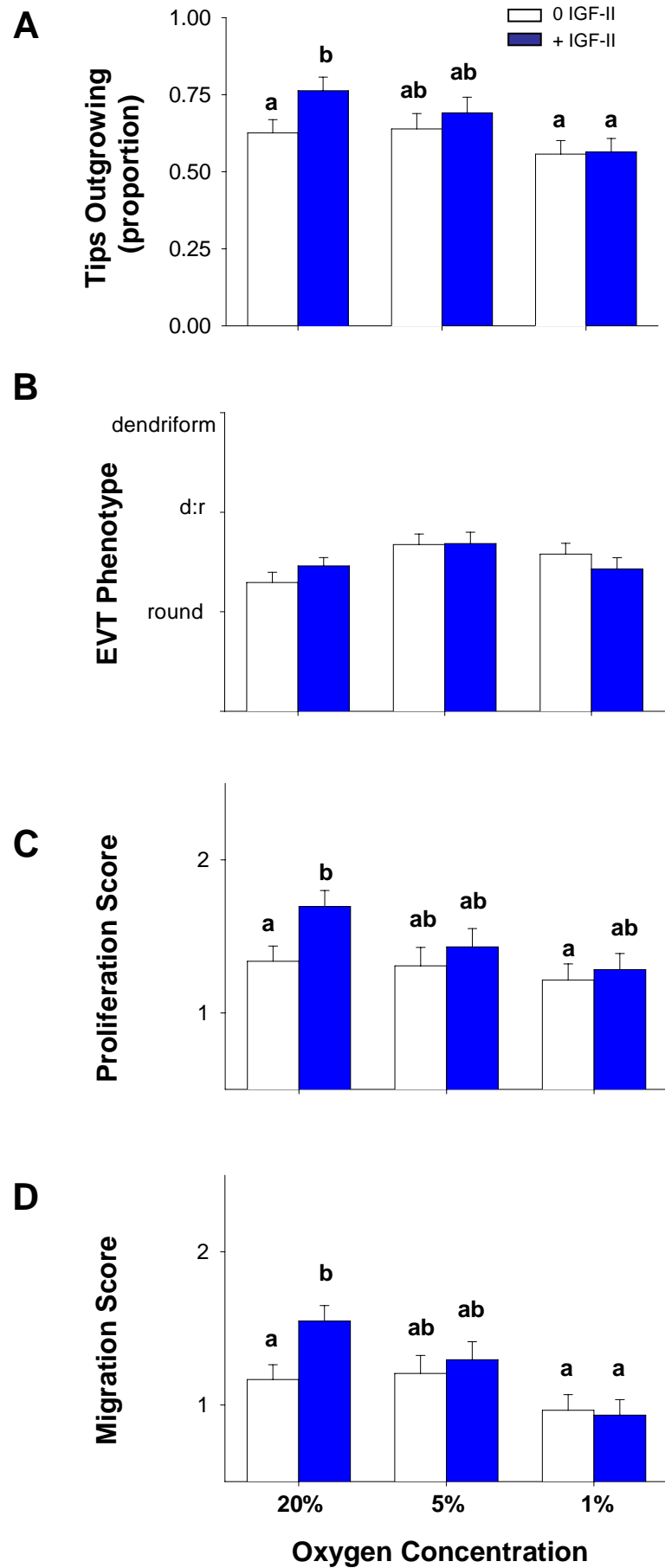
Given there was an effect of exogenous IGF-II on outgrowth in explants cultured under 20% oxygen regardless of gestational age, and the gestational age affected explant growth in culture, we determined whether there was a differential response of explants to exogenous IGF-II treatment with gestational age. There was no effect of gestational age on the proportion of tips growing per explant in response to IGF-II treatment (Figure 3.7a). However, there was a clear difference in the proliferative response of explants to exogenous IGF-II between placenta at 7 and 8 weeks of gestation with those at 9, 10 and 12 weeks (Figure 3.7b). There was also a dramatic increase in the migratory response of explants to



**Figure 3.5 Effect and interaction of oxygen and IGF-II concentrations on villous explant outgrowth at 7-8 weeks of gestation.** First trimester human villous explants were assessed for outgrowth and migratory behaviour on day 6 of culture under 1%, 5% or 20% oxygen without (0 IGF-II) and with the addition of 17nmol IGF-II (+ IGF-II). Villous tip outgrowth was expressed as the proportion of tips bearing cell columns per explant (a). Arbitrary scores were used to assess the extravillous cytotrophoblast phenotype as being round, dendriform or a mixture of the two (d:r) (b). Extent of proliferation observed at the tips of villi (c) and the extent of extravillous cytotrophoblast migration (d) were also recorded using arbitrary scores. Data are expressed as mean  $\pm$  SEM. n = 8 placentae, 8 wells/treatment/placenta. Compared with 20% oxygen no IGF-II, \*\*P < 0.047 and \*P < 0.001, determined by Mann-Whitney U Tests.



**Figure 3.6 Effect and interaction of oxygen and IGF-II concentrations on placental villous explant outgrowth at 9-13 weeks of gestation.** First trimester human villous explants were assessed for outgrowth and migratory behaviour on day 6 of culture under 1%, 5% or 20% oxygen with and without the addition of 17nmol IGF-II. Villous tip outgrowth was expressed as the proportion of tips bearing cell columns per explant (a). Arbitrary scores were used to assess the extravillous cytotrophoblast phenotype as being round, dendriform or a mixture of the two (d:r) (b). Extent of proliferation observed at the tips of villi (c) and the extent of extravillous cytotrophoblast migration (d) were also recorded using arbitrary scores. Data are expressed as mean  $\pm$  SEM. n = 8 placentae, 8 wells/treatment/placenta. Different letters denote statistical difference, where a vs b,  $P < 0.047$ , as determined by Mann-Whitney U Tests.



exogenous IGF-II between placentas at 12 weeks of gestation compared with the 7-11 weeks (Figure 3.7 c).

### 3.2.1.6 Effect of oxygen and exogenous IGF-II on villous gene expression at 7-8 weeks of gestation

To determine whether the effect of IGF-II and oxygen concentration on villous explant outgrowth at 7-8 weeks of gestation coincided with alterations in gene expression, *Igf2*, *Igf1r*, *Igf2r*, *Upar* and *Tgfβ1* mRNA were quantitated relative to *18s* rRNA using sequence-specific primers and Real Time RT-PCR. All five genes were expressed in villous explant cytotrophoblasts (Table 3.1) and produced the expected exponential curves of fluorescence. However, there was considerable variation in gene expression between samples, regardless of treatment group.

Bands visualised after gel electrophoresis revealed a single cDNA product was amplified with each primer pair, all of which were consistent with the predicted sizes of *18s* (91 bp), *Igf2* (90 bp), *Igf2r* (88 bp), *Igf1r* (142 bp), *Upar* (71 bp), *Tgfβ1* (104 bp) (Figure 3.8). The expression of *18s* rRNA did not vary significantly across the six treatments studied (data not shown). As  $\Delta$ CT is inversely related to levels of mRNA expression, the abundance of *Igf2* mRNA transcripts relative to *18s* rRNA (mean  $\Delta$ CT  $\pm$  SEM =  $2.15 \pm 0.3$ ) was significantly higher than *Igf1r* ( $10.9 \pm 3.3$ ), *Igf2r* ( $11.9 \pm 2.2$ ), *Upar* ( $12.6 \pm 3.4$ ) and *Tgfβ1* ( $10.4 \pm 4.0$ ) mRNA in explant cytotrophoblasts across all treatments ( $p < 0.001$ , Table 3.1). Expression of all genes were normalised to the house-keeping gene, *18s* rRNA and expressed as a percent of the mean value for the 20% oxygen without exogenous IGF-II treatment group.

Culture under 1% oxygen increased the expression of *Igf2* mRNA by 3.5-fold and 4-fold compared with 20% ( $P=0.04$ ) and 5% oxygen explants. However, this did not reach statistical significance with 5% oxygen explants ( $P = 0.065$ ) (Figure 3.9a). Exogenous IGF-II did not affect the expression of *Igf2* mRNA in explants cultured under 20% or 5% oxygen. However, there appeared to be a negative effect of IGF-II on gene expression in 1% oxygen explants, where the addition of exogenous IGF-II reduced *Igf2* mRNA expression by nearly 65%, although this failed to reach statistical significance ( $P= 0.06$ , Figure 3.9 a).

There was no effect of low oxygen concentration on *Igf2r* gene expression by villous explants compared with those cultured in 20% oxygen (Figure 3.9b). There was, however, a 9-fold difference in *Igf2r* mRNA expression between 1% and 5% oxygen explants, where the transcript was significantly more abundant

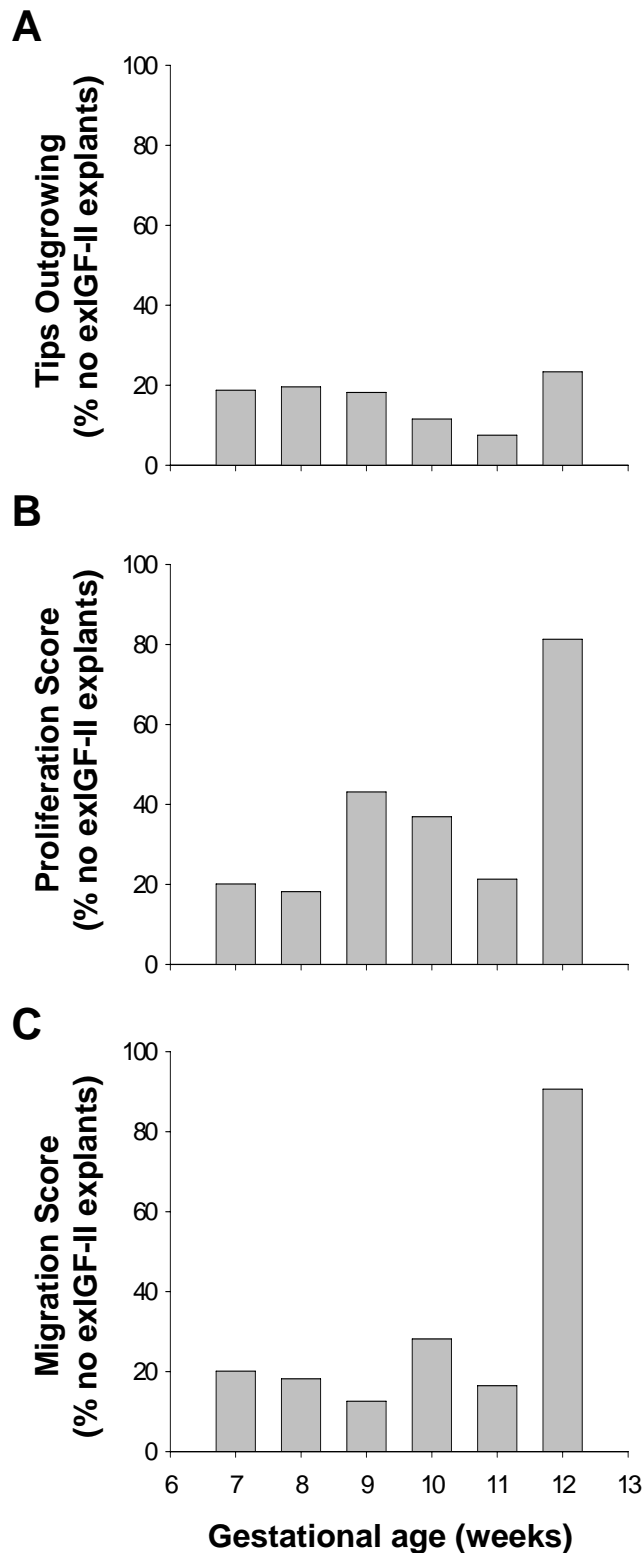


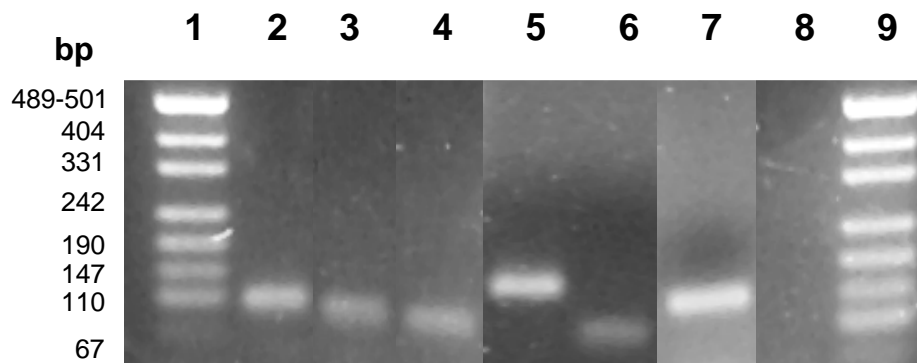
Figure 3.7 Association of gestational age with the growth response of placental villous explants to exogenous IGF-II. Villous explant outgrowth in response to exogenous IGF-II in 20% oxygen atmospheres, across gestational age was expressed as a percentage of the explants cultured in the absence of exogenous IGF-II (exIGF-II, as represented by zero line). Growth responses were indicated by the proportion of villous tips bearing cell columns per explant (a) and the extent of proliferation (b) and migration (c) from those tips. Figures represent the mean differential response within each placenta  $\pm$  SEM.

Table 3.1 Real Time PCR assay characteristics

Gene	Lowest Ct	Highest Ct	$\Delta Ct^*$	Amplification efficiency (%)
<i>18s</i>	14.7	19.8	-	76
<i>Igf2</i>	16.6	25.7	2.15 $\pm$ 0.3	87
<i>Igf1r</i>	21.9	29.9	10.9 $\pm$ 3.3	98
<i>Igf2r</i>	26.0	33.0	11.9 $\pm$ 2.2	73
<i>Upar</i>	24.2	32.8	12.6 $\pm$ 3.4	82
<i>Tgf<math>\beta</math>1</i>	21.86	36.2	10.4 $\pm$ 4.0	85

Number of explants = 48, from each of 8 placentas). Ct = cycle threshold,  $\Delta Ct = 18s - gene\ of\ interest$ .

\* mean  $\Delta Ct \pm SEM$ , inversely related to mRNA expression.



**Figure 3.8 PCR amplification products of sequence specific primers.** Lanes 1 and 9 show molecular weight markers with size in base pairs (bp) denoted. Lanes 2-7 show, 18S (91 bp), IGF-II (90 bp), IGF2R (88 bp), IGF1R (142 bp), uPAR (71 bp), TGF- $\beta$ 1 (104 bp) PCR amplification products, respectively. Lane 8 shows representative no cDNA template control.



in 1% oxygen explants ( $P = 0.003$ , Figure 3.9b). There was no effect of exogenous IGF-II on *Igf2r* gene expression. When comparing all treatment groups, *Igf2r* mRNA expression was increased 2-fold in explants grown in 1% oxygen compared with those exposed to 20% oxygen and treated with IGF-II ( $P = 0.04$ , Figure 3.9b).

There was no significant effect of oxygen concentration or exogenous IGF-II on *Igf1r*, *Upar* or *Tgfβ1* gene expression (Figure 3.9c-e).

#### *3.2.1.7 Effect of oxygen and exogenous IGF-II on placental villous explant IGF-II protein secretion at 7-8 weeks of gestation*

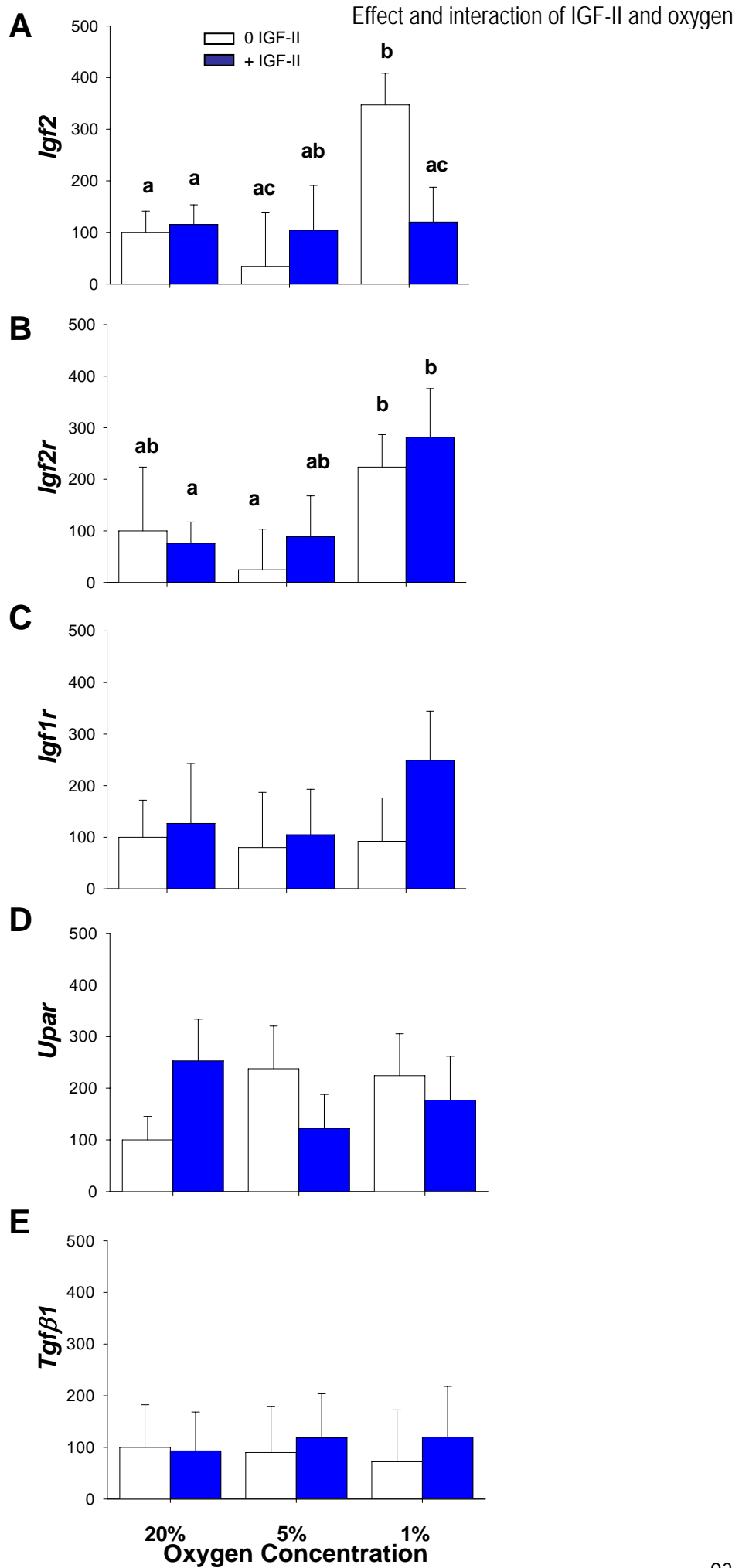
Culture of villous explants in 1% oxygen atmospheres increased IGF-II protein secretion by 10-fold compared with 20% oxygen explants (mean  $\pm$  SEM =  $9.85 \pm 2.74$  versus  $0.98 \pm 0.67$  ng/ml, respectively,  $P < 0.005$ ).

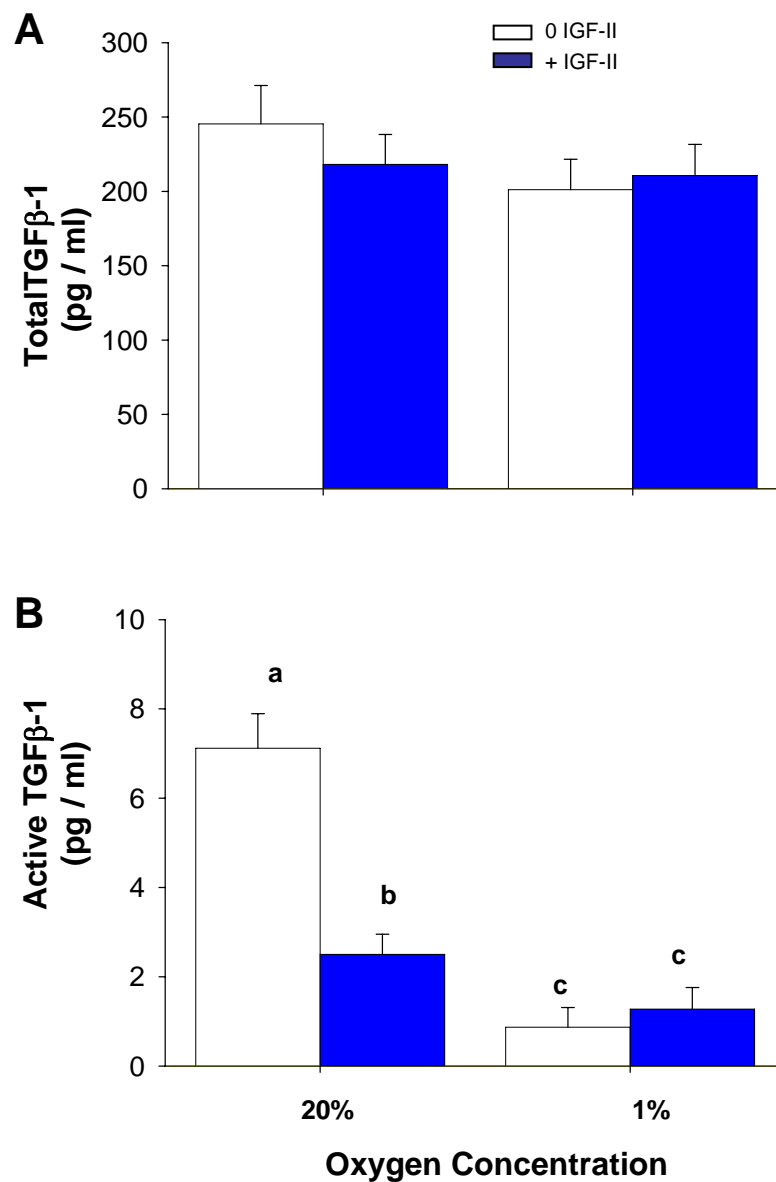
#### *3.2.1.8 Effect of oxygen concentration and exogenous IGF-II on TGFβ1 activation at 7-8 weeks of gestation*

As the TGF-β1 protein requires activation to have biological effects, the effects of oxygen concentration and exogenous IGF-II on TGFβ1 activation was determined in villous explant-conditioned media.

Total TGF-β1 protein secretion (inactive + active) was unaltered by culture in 1% oxygen or exposure to exogenous IGF-II (Figure 3.10a), which was consistent with gene expression studies. Culture of villous explants in 1% oxygen atmospheres reduced the concentration of active TGF-β1 by 90%, compared with 20% oxygen explants (mean  $\pm$  SEM =  $0.88 \pm 0.44$  versus  $7.12 \pm 0.77$  pg/ml, respectively,  $P < 0.005$ , Figure 3.10b). There was no effect of IGF-II treatment on the concentration of active TGF-β1 in explants exposed to 1% oxygen (Figure 3.10b). However, exogenous IGF-II reduced the concentration of active TGF-β1 protein by 65% in explants cultured in 20% oxygen (mean  $\pm$  SEM =  $2.50 \pm 0.46$  versus  $7.12 \pm 0.77$  pg/ml, respectively,  $P < 0.005$ ) (Figure 3.10b). Despite reductions in TGF-β1 activation with the addition of IGF-II to explants cultured under 20% oxygen atmospheres, concentrations of active TGF-β1 were still significantly higher than in 1% oxygen cultures ( $P < 0.05$ , Figure 3.10b).

**Figure 3.9 Effect and interaction of oxygen and IGF-II concentrations on placental villous explant gene expression.** *Igf2* (a), *Igf2r* (b), *Igf1r* (c), *Upar* (c) and *Tgfβ1* (c) gene expression were measured in cytotrophoblasts of first trimester (7-8 weeks gestation) human placental villous explants on day 6 of culture under 1%, 5% or 20% oxygen with and without the addition of 17nmol IGF-II using Real-Time RT-PCR. Data were normalised to the housekeeper, 18s rRNA, and each treatment expressed as a percentage of the mean value for the 20% oxygen, no exogenous IGF-II treatment group, using the  $2^{-\Delta\Delta CT}$  method. Data are expressed as mean  $\pm$  SEM. n = 8 placentae, 8 wells pooled per treatment per placenta. Different letters denote statistically significant difference as determined by Mann-Whitney U Tests; a vs b,  $P < 0.04$  and b vs c,  $P < 0.065$ .





**Figure 3.10 Effect and interaction of oxygen and IGF-II concentration on villous explant TGF- $\beta$ 1 production and activation.** Total (inactive + active, a) and active (b) TGF- $\beta$ 1 protein concentrations were determined in first trimester (7-8 weeks gestation) human villous explant-conditioned media on day 6 of culture under 1% or 20% oxygen with and without the addition of 17nmol IGF-II using a commercially available TGF- $\beta$ 1 ELISA. A four parameter logistic regression curve was used to determine the concentration of protein, after which, media blanks were subtracted from values. Data are expressed as mean pg / ml  $\pm$  SEM. n = 12-13 placentas, 1 representative well/treatment/placenta. Superscripts denote statistical significance determined using Mann-Whitney U Test compared with a, P < 0.005 or b versus c, P < 0.05.

3.2.1.9 Correlations of gene expression and TGF- $\beta$ 1 activation with placental villous explant outgrowth at 7-8 weeks of gestation

All villous outgrowth, gene expression and protein studies were undertaken in each placental sample. Therefore, we investigated whether there were any significant correlations between these parameters (Table 3.2). *Igf2* and *Igf2r* gene expression were positively associated with villous proliferation ( $r = 0.43$ ,  $P = 0.003$  and  $r = 0.38$ ,  $P = 0.008$ , respectively) and migration ( $r = 0.47$ ,  $P = 0.001$  and  $r = 0.39$ ,  $P = 0.007$ , respectively). *Igf2* transcription was also positively correlated with the proportion of tips that were outgrowing per explant ( $r = 0.44$ ,  $P = 0.003$ ). In contrast, total TGF $\beta$ 1 protein was negatively correlated with migratory phenotype ( $r = -0.25$ ,  $P = 0.03$ ) and active TGF $\beta$ 1 was negatively correlated with villous tip outgrowth and migration ( $r = -0.59$ ,  $P < 0.001$  and  $r = -0.30$ ,  $P = 0.034$ , respectively). There were no significant correlations between morphological parameters and *Upar*, *Igf1r* or *Tgf $\beta$ 1* gene expression or surprisingly, with IGF-II protein secretion. In placental villous conditioned media, IGF-II protein was negatively correlated with concentrations of active TGF- $\beta$ 1 protein ( $r = -0.42$ ,  $P = 0.003$ ).

**Table 3.2 Association of gene expression, TGF- $\beta$ 1 protein secretion and villous explant outgrowth**

	Tips growing	EVT phenotype	Proliferation	Migration
<i>Igf2</i>	0.44**	0.12	0.43**	0.47**
<i>Igf2r</i>	0.17	-0.21	0.38*	0.39*
<i>Igf1r</i>	0.15	0.17	-0.04	0.16
<i>Upar</i>	-0.01	0.05	-0.05	0.09
<i>Tgf<math>\beta</math>1</i>	0.07	0.22	-0.21	0.16
Active TGF- $\beta$ 1 <sup>#</sup>	-0.59**	-0.30*	-0.13	-0.12
Total TGF- $\beta$ 1	0.09	-0.25*	-0.05	0.16
IGF-II <sup>#</sup>	0.23	0.03	0.19	0.28

Table displaying correlation coefficients ( $r$ ), which were significant at \*  $P < 0.04$  or \*\*  $P < 0.003$ , as determined by Pearson's bi-variate correlation analyses. # Negatively correlated with each other,  $r = -0.42$   $P = 0.003$ . Across all treatments, with  $n = 48$  samples from 8 placenta.

### 3.3 Discussion

The low-oxygen environment in which human placental development occurs during the first trimester of pregnancy is believed to be important in controlling EVT outgrowth (Caniggia and Winter 2002; Genbacev, Zhou *et al.* 1997) and is essential for pregnancy success (Hustin, Jauniaux *et al.* 1990; Jauniaux, Greenwold *et al.* 2003; Jauniaux, Zaidi *et al.* 1994). In the present study, we have demonstrated for the first time that this low oxygen environment during early pregnancy, stimulates human placental outgrowth, induces *Igf2* gene expression and inhibits TGF- $\beta$ 1 activation. IGF-II appears to mimic the low oxygen response, as addition of exogenous IGF-II to placental explants cultured in 20% oxygen stimulates placental outgrowth and inhibits TGF- $\beta$ 1 activation. These suggest that prior to the onset of maternal blood flow to the intervillous space, IGF-II mediates the low oxygen response during early pregnancy to promote placental invasion. Later in pregnancy, when oxygen concentrations increase, placental invasion declines and differentiation of the placental exchange region occurs to increase functional capacity, potentially via TGF- $\beta$ 1 activity.

Placental development requires spatially and temporally regulated invasion of the maternal decidua and its vasculature by EVTs. This process requires rapid changes in the expression repertoire of adhesion molecules by the villous cytotrophoblast, a highly organised and polarised epithelium, into a phenotype that is characteristic of endothelial cells and leukocytes and capable of invasion (epithelial to mesenchymal transformation). In the current study, culture of 7-8 weeks gestation placental explants in 5% or 1% oxygen, which is similar to the assumed physiological oxygen environment at the fetomaternal interface *in vivo* (Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992), increased villous tip outgrowth and the proportion of EVTs displaying a migratory appearance, compared with 20% oxygen atmospheres. This data is in agreement with studies using isolated cytotrophoblast cells and villous explant culture systems demonstrating that oxygen can influence cell-matrix interactions, cell-cell associations, cell cycle progression and initial cellular differentiation along the invasive pathway. For instance, previous investigations of 5-8 week old villous explants exposed to hypoxia (2% oxygen; ~14 mmHg) have demonstrated an inhibition of cytotrophoblast terminal differentiation into syncytium (Alsat, Wyplosz *et al.* 1996), increased trophoblast proliferation/outgrowth at tips of anchoring villi (Caniggia 2000; Genbacev, Joslin *et al.* 1996) through direct impacts on cell cycle regulatory proteins (Genbacev, Zhou *et al.* 1997), increased HLA-G and  $\alpha$ 5 integrin expression (Iwaki, Yamamoto *et al.* 2004; Kilburn, Wang *et al.* 2000) and the activities of MMP-2 (Caniggia, Lye *et al.* 1997; Caniggia, Taylor *et al.* 1997) and focal adhesion kinase (MacPhee, Mostachfi *et al.* 2001; Seko, Takahashi *et al.* 1999).

In the present study, there was no effect of 5% or 1% oxygen on proliferation or migration scores, despite a higher proportion of EVTs displaying a migratory, dendriform phenotype in explants exposed to low oxygen phenotype. This suggests that low oxygen initiates the first stages of the extravillous differentiation pathway, but may not be sufficient for trophoblast completion of the invasion path. Additionally, our data may indicate that the explants were already at their maximal outgrowth capacity given that the extended culture period of six days without media change.

Independent of oxygen concentration, the extent of villous tip proliferation and migration declined progressively with gestational age of the tissue, suggesting that trophoblast is innately programmed to proliferate and invade optimally in early stages of gestation, when maternal blood perfusion of the placenta is minimal. Indeed, other investigators have shown that exposure to hypoxia stimulates first trimester immortalised human trophoblast invasion by more than 40% and is associated with reduced secretion of tissue inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2 and increased uPAR (and therefore uPA cell surface-associated activity) expression compared with high (20% oxygen) (Canning, Postovit *et al.* 2001; Graham, Fitzpatrick *et al.* 1998). More quantitative methods to measure proliferation and migration in response to oxygen rather than arbitrarily scoring placental villous explants are required to confirm and supplement the current observations. This may involve measuring tritiated thymidine analogue uptake by villous explants in response to low oxygen concentration or measuring distance and area of resultant explant outgrowth using Video Image Analysis software on photomicrographs.

In the present study, exposure of 7-8 weeks gestation villous explants to low oxygen atmospheres almost completely abolished activation of TGF- $\beta$ 1. Explant synthesis of total and active TGF- $\beta$ 1 protein was negatively correlated with villous tip outgrowth. This is consistent with previous investigations demonstrating that TGF- $\beta$ 1 is negative regulator of first trimester trophoblast cell proliferation (Graham and Lala 1992), migration (Irving and Lala 1995) and invasion (Graham and Lala 1991; Graham and Lala 1992) *in vitro*. TGF- $\beta$ 1 reduces trophoblast migration and invasion by increasing the expression of TIMPs (Graham and Lala 1991; Khoo, Bechberger *et al.* 1998), plasminogen activator inhibitor (PAI)-1 (Graham 1997), promotes intercellular adhesion by increasing E-cadherin expression (Karmakar and Das 2004), promotes extracellular matrix deposition (Chen, Yang *et al.* 2005; Feinberg, Kliman *et al.* 1994) and reduces uPA (Graham and Lala 1991) and MMP-9 activation (Meisser, Chardonnens *et al.* 1999). Furthermore, expression of the TGF- $\beta$  superfamily transmembrane protein, endoglin also inhibits

human trophoblast outgrowth and may be necessary for the inhibitory effects of TGF- $\beta$  (Caniggia, Taylor *et al.* 1997). Interestingly, the endoglin gene contains a functional hypoxia response element (HRE) (Sanchez-Elsner, Botella *et al.* 2002). However, whether endoglin expression was altered by oxygen concentration in the current study has yet to be established.

In the current study, the mechanisms by which hypoxia decreases activation of TGF- $\beta$ 1 protein did not involve inhibition of *Tgf $\beta$ 1* transcription nor total protein synthesis. This is in contrast to *Tgf $\beta$ 3* gene regulation by hypoxia in placental villous explants (Caniggia 2000). Enzymatic activation of TGF- $\beta$ 1 *in vivo* is mediated by glycosidases, serine proteases and thrombospondin-1 (Gleizes, Munger *et al.* 1997; Munger, Harpel *et al.* 1997). Studies in human monocytes and more recently in endothelial cells have also revealed that activation of TGF- $\beta$ 1 occurs on the IGF2R. Upon binding of LTGF- $\beta$ 1 to the IGF2R (due to M6P residues in the latency-associated peptide), IGF2R simultaneously complexes with the uPAR on the cell surface (Godar, Horejsi *et al.* 1999; Leksa, Godar *et al.* 2005). The binding of uPA to uPAR allows uPA to cleave plasminogen into plasmin, the latter of which cleaves the latency-associated peptide and allows release of biologically active TGF- $\beta$ 1 (Godar, Horejsi *et al.* 1999; Leksa, Godar *et al.* 2005). Previous studies have failed to demonstrate an effect of oxygen concentration on TGF- $\beta$ 1 activation in human fibroblast cultures isolated from term placenta (Chen, Yang *et al.* 2005). This may reflect differences in the composition of culture media used in our villous explant system. Our media is supplemented with plasminogen, which is required for activation of TGF- $\beta$ 1 by the IGF2R complex (Godar, Horejsi *et al.* 1999; Leksa, Godar *et al.* 2005) and is secreted by trophoblasts as they invade *in vivo* (Graham, Postovit *et al.* 2000). Additionally, fibroblasts may not express all components of the IGF2R-uPAR complex.

To elucidate the effects of hypoxia on TGF- $\beta$ 1-IGF2R-uPAR activation complex, we quantified the expression of *Upar*, *Igf2r* and *Igf1r* genes. Alterations in the expression of the latter may have indirectly influenced IGF2R ligand occupancy. All three genes were expressed by outgrowing trophoblast in our explants. In addition, there was a trend for increased *Igf2r* transcription in explants exposed to 1% oxygen atmospheres compared with 20% but this failed to reach significance. There was no effect of oxygen concentration on explant expression of *Upar* mRNA. These are in contrast to previous findings, which demonstrated an induction of this gene in response to hypoxia in the transformed cell line, HTR-8/SVneo (Graham, Fitzpatrick *et al.* 1998). A lack of an effect of oxygen availability on gene expression may have been due to differences in study design and inherent differences in trophoblast populations studied. Furthermore, in the current investigation, the postulated effect of oxygen concentration on gene



expression may have been diluted as analyses were performed on pooled samples of RNA per placenta, per treatment and we observed significant variations in growth of individual explants within treatments and between patients. Additionally, the heterogeneous nature of the outgrowing trophoblasts used for analysis may have been responsible for the considerable variation in gene expression across treatments. Isolating certain trophoblast populations in our explants, ie those that constitute the cell column or those that have detached and are migrating and invading could provide more informative data on gene expression.

Alternatively, differences in the length of hypoxic exposure may underlie the inconsistencies between the current and previous studies. Previous studies only acutely exposed their cells to hypoxia – for a duration of 24 hours. However, in the current study, explants were exposed to prolonged hypoxia, that is, for 6 days. It is well established that acute and chronic hypoxic exposure can have different effects on transcription and cellular function (Uchida, Rossignol *et al.* 2004). Further mRNA analyses of outgrowing trophoblasts from explants after different lengths of hypoxic exposure may help to elucidate this.

Importantly, in the current investigation, explants prepared from placentas at 7-8 weeks of gestation and exposed to 1% oxygen, displayed increased *Igf2* gene expression and protein secretion, compared to those exposed to 20% oxygen atmospheres. Addition of exogenous IGF-II appeared to inhibit endogenous *Igf2* transcription in explants exposed to 1% oxygen atmospheres. This negative feedback has been demonstrated in differentiating myoblasts, where it appears to operate through the IGF1R (Magri, Benedict *et al.* 1994). As there was no effect of exogenous IGF-II on explant endogenous *Igf2* transcription in 5% or 1% oxygen atmospheres, this negative feedback may operate in a concentration dependent manner.

Although a defined HRE (HIF-1 binding site) in the upstream region of the *Igf2* gene remains to be identified, other investigators have reported that *Igf2* is induced by hypoxia in some cell types, including mouse embryonic fibroblasts, rat osteoblasts and astrocytes, human hepatocarcinoma (HepG2) and bovine pulmonary endothelial cells (Feldser, Agani *et al.* 1999; Kim, Bae *et al.* 1998; Steinbrech, Mehrara *et al.* 2000; Tucci, Nygard *et al.* 1998). In the current study, IGF-II secretion was negatively correlated with explant activation of TGF- $\beta$ 1 and IGF-II treatment of villous explants incubated in 20% oxygen atmospheres inhibited TGF- $\beta$ 1 activation (to a similar extent to that seen in 1% oxygen explants). This suggested that IGF-II may mediate the low oxygen response on TGF- $\beta$ 1 activation.

The mechanism for IGF-II inhibition of TGF- $\beta$ 1 activation is predicted to involve competitive interaction between these two factors with IGF2R. Although the IGF2R binding sites for LTGF- $\beta$ 1 (mannose-6 phosphate site) and IGF-II are distinct, IGF2R-IGF-II interaction induces a conformational change in the structure of IGF2R, which causes the LTGF- $\beta$ 1 binding site to become inaccessible (Leksa, Godar *et al.* 2005). Furthermore, other studies performed in the laboratory have supported the notion that IGF-II has a greater affinity for IGF2R than LTGF- $\beta$ 1 (Roberts, CT unpublished). Given that IGF2R and uPAR are expressed by trophoblast (confirmed in this study), which also up-regulate IGF-II synthesis in 1% oxygen atmospheres, IGF-II is likely to saturate the IGF2R, thereby preventing TGF- $\beta$ 1 activation. This is especially likely since *Igf1r* transcription was not affected by oxygen.

*In vivo*, IGF-II is highly expressed by the human placenta but declines by late gestation (Han, Bassett *et al.* 1996). IGF-II is most highly expressed by EVT's deep in the decidua or within and surrounding maternal uterine spiral arterioles and lowest in cells of the villus (Han, Bassett *et al.* 1996; Ohlsson, Holmgren *et al.* 1989; Ohlsson, Larsson *et al.* 1989). These suggest that although invading trophoblasts encounter a gradient of increasing oxygen concentration as they approach the maternal vasculature, they continue to synthesise IGF-II. Additionally, IGF-II expression in the placenta remains high (Han, Bassett *et al.* 1996) following flow of maternal blood into the intervillous space (Jaffe and Woods 1993; Jauniaux, Jurkovic *et al.* 1992; Jauniaux, Watson *et al.* 2000) and elevated local oxygen concentrations ((Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992), suggesting little regulatory effect of oxygen on IGF-II synthesis *in vivo*. Despite the *in vitro* findings of the current work, this would suggest that, *in vivo*, IGF-II synthesis is not solely regulated by oxygen availability. Interestingly, IGF-II has been demonstrated to increase HIF-1 $\alpha$  protein expression, HIF-1 DNA binding activity, and transactivation of target genes under normoxic atmospheres *in vitro* (Feldser, Agani *et al.* 1999; Zelzer, Levy *et al.* 1998). Therefore, IGF-II may extend HIF actions in the placenta, including effects on *Igf2* gene expression in trophoblasts as they invade the decidua and subsequent to maternal blood perfusion of the placenta.

As IGF-II inhibited TGF- $\beta$ 1 activation, a negative regulator of trophoblast function, exogenous IGF-II would be expected to increase villous explant outgrowth. In the current investigation, treatment of villous explants cultured in 20% oxygen with IGF-II, also increased villous tip outgrowth. Regardless of the gestational age of placental villous explants, exogenous IGF-II increased the proportion of villous tips outgrowing and in explants greater than 9 weeks of gestation, stimulated trophoblast proliferation and migration from those tips. Furthermore, stimulation of villous tip outgrowth by exogenous IGF-II

treatment in explants at 7-8 weeks of gestation cultured in 20% oxygen, resembled outgrowth seen in explants exposed to low oxygen atmospheres, suggesting that IGF-II may mediate the growth promoting effects of low oxygen availability. This is consistent with previous studies that demonstrated the positive effects of exogenous IGF-II on first trimester placental trophoblast DNA synthesis and proliferation (Ohlsson, Holmgren *et al.* 1989), EVT cell migration (Irving and Lala 1995; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001) and invasion (Hamilton, Lysiak *et al.* 1998) *in vitro*. Interestingly, IGF-II also promotes differentiation of murine trophoblast into invasive cells, by transforming ectoplacental cone cells (early trophoblast stem cells) into invasive trophoblast giant cells *in vitro* (Kanai-Azuma, Kanai *et al.* 1993).

The effects of IGF-II on placental explant outgrowth under 20% oxygen atmospheres may have been mediated through any of the three cell surface receptors, IGF1R, IGF2R and InsR (Abu-Amero, Ali *et al.* 1998; Desoye, Hartmann *et al.* 1997; Fang, Furesz *et al.* 1997; Holmes, Porter *et al.* 1999; Jones, Hartmann *et al.* 1993; Milio, Hu *et al.* 1994; Ohlsson, Holmgren *et al.* 1989), which are expressed by human placental trophoblast. IGF1R-InsR hybrids are also expressed by the human placenta (Soos, Field *et al.* 1993), however their contribution to IGF-II effects in our explant system are unknown. We predict that IGF-II stimulation of trophoblast proliferation in our explants occurred via IGF1R, which activates the insulin receptor substrate-1-dependent intracellular signalling pathways (Jones and Clemmons 1995). Additionally, in the presence of abundant IGF-II, InsR has been demonstrated to mediate IGF-II mitogenic effects (Morrione, Valentinis *et al.* 1997). Importantly, it has been reported that IGF-II promotion of invasion is independent of IGF1R *in vitro* (Hamilton, Lysiak *et al.* 1998). As IGF-II preferentially binds to IGF2R (Jones and Clemmons 1995), the latter may play a dominant role in IGF-II actions on explant outgrowth.

As well as its established role in IGF-II clearance, several reports indicate that IGF2R also functions in IGF-II signalling (Groskopf, Syu *et al.* 1997; Ikezu, Okamoto *et al.* 1995; Ikushima, Munakata *et al.* 2000; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001; Minniti, Kohn *et al.* 1992; Nishimoto 1993; Tsuruta, Eddy *et al.* 2000; Zhang, Tally *et al.* 1997). Interaction of IGF-II with IGF2R, can stimulate an intracellular signalling pathway using a G<sub>i</sub>-coupled receptor (Ikezu, Okamoto *et al.* 1995; Murayama, Okamoto *et al.* 1990) and mitogen activated protein kinase (MAPK) (Groskopf, Syu *et al.* 1997; Ikezu, Okamoto *et al.* 1995; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001; Nishimoto 1993), inducing cell differentiation, migration and invasion. Furthermore, activation of plasmin on uPAR-IGF2R complex can activate latent MMPs, which are also involved in cellular migration and invasion. Consistent with this, exogenous IGF-II increased MMP-2 and MMP-9 activity in

first trimester primary trophoblast cultures (Hills, Elder *et al.* 2004). Indeed, in the current study, *Igf2* and *Igf2r* gene expression were positively correlated with villous tip outgrowth, and are both abundantly expressed by EVT at the invading front (Han, Bassett *et al.* 1996; Ohlsson, Holmgren *et al.* 1989), further supporting a role for IGF2R in mediating IGF-II regulation of trophoblast migration and invasion. Interestingly, exogenous IGF-II increased the proliferation and migration response by 60-70% in explants exposed to 20% oxygen atmospheres in placenta from 12 weeks gestation. This is likely to reflect a stimulation in growth of explants that are otherwise not growing well as there was a decline in outgrowth with gestational age. Certainly, IGF-II has well documented roles in the promotion of cell survival and protection from apoptosis (Jones and Clemmons 1995).

Despite the positive effects of low oxygen availability on explant outgrowth from placenta at 7-8 weeks of gestation, culture of explants from greater than 9 weeks of gestation in low oxygen atmospheres reduced the proportion of villous tips outgrowing. This observation presumably reflected a state of hypoxic stress, as it is believed that endovascular trophoblast plugs, which initially occlude the maternal spiral arteries, gradually disperse from 10 weeks of gestation, allowing maternal arterial blood to flow into the intervillous space and increased oxygen tension to 8-9% (Jaffe, Jauniaux *et al.* 1997; Jauniaux, Hempstock *et al.* 2003; Jauniaux, Jurkovic *et al.* 1992). Comparison of explant outgrowth in response to 5% or 1% oxygen with 20% oxygen, revealed there was a significant effect of oxygen tension in placenta of 11 weeks and 10 weeks gestation, respectively. This was shown as a gradual decline in the effect of oxygen concentration as gestational age increased, supporting the concept that placenta becomes exposed to increasing oxygen concentrations late in the first trimester of pregnancy *in vivo*. Furthermore, the change in responsiveness to low oxygen atmospheres in explants greater than 10 weeks gestation in 1% oxygen is consistent with the observation that endovascular trophoblastic plugs dissociate at this time (Burton, Jauniaux *et al.* 1999; Jaffe, Jauniaux *et al.* 1997; Jauniaux, Hempstock *et al.* 2003; Jauniaux, Jurkovic *et al.* 1992; Jauniaux, Watson *et al.* 2001; Rodesch, Simon *et al.* 1992). The decline in explant outgrowth in placenta less than 11 weeks of gestation in 5% oxygen is also expected, as this concentration of oxygen is still lower than that reported for the intervillous space *in vivo*, which is 8-9% oxygen at this time (Rodesch, Simon *et al.* 1992).

In later gestation, the placenta undergoes dramatic remodelling and differentiation to increase its functional efficiency and support the demands of the growing fetus. These changes result from developmentally regulated periods of villous arborization, branching and non-branching angiogenesis and trophoblast terminal differentiation into syncytium (Kaufmann, Mayhew *et al.* 2004), the latter of which is specialised for hormone synthesis. These all result in increased surface area of

syncytiotrophoblast for placental exchange between the maternal and fetal circulations. Perturbations in the normal pattern of placental development will lead to a placenta with altered function and may compromise the pregnancy (Mayhew, Charnock-Jones *et al.* 2004). Interestingly, TGF- $\beta$ 1 promotes terminal differentiation of trophoblast into syncytium (Graham, Lysiak *et al.* 1992) by increasing cadherin-11 cell surface expression (Getsios, Chen *et al.* 1998). Thus, we hypothesize that later in pregnancy, when IGF-II synthesis declines (Han, Bassett *et al.* 1996) and the placenta is well oxygenated, trophoblast activation of TGF- $\beta$ 1 might provide a means for functional morphogenesis of the placental villous tree. Certainly, TGF- $\beta$  plays dominant roles in regulating the development and patterning (by determining the number of branches, branch angles, and morphological features of the epithelial stalk) of other organs composed of epithelial trees, including the mammary gland (Ewan, Shyamala *et al.* 2002; Pollard 2001; Silberstein 2001), lung (Karihaloo, Nickel *et al.* 2005) and kidney (Bartram and Speer 2004; Bush, Sakurai *et al.* 2004; Davies and Fisher 2002; Karihaloo, Nickel *et al.* 2005). Trophoblast activation of TGF- $\beta$ 1 in precise positions on the chorionic villous tree may therefore modify the morphogenesis and complexity of the arborizing structure in late pregnancy.

In conclusion, the current and previous studies have indicated that oxygen availability at the fetomaternal interface “fine-tunes” trophoblast behaviour by influencing the balance of IGF-II expression and TGF- $\beta$ 1 activation. We propose that early in pregnancy, prior to the onset of maternal blood flow to the intervillous space, the hypoxic environment stimulates IGF-II synthesis, which promotes trophoblast villous outgrowth and invasion of the maternal decidua by direct effects on trophoblast function and inhibiting molecules secreted by the decidua, namely TGF- $\beta$ 1, which act to inhibit this process. This promotes circumferential growth of the placenta and allows adequate invasion and subsequent remodelling of the maternal vasculature. Later in pregnancy, when oxygen levels increase and IGF-II synthesis declines, TGF- $\beta$ 1 sequestered in the decidua can act on trophoblast to promote terminal differentiation of EVT, which is required for functional capacity. It is possible that alterations in the expression of IGF-II, TGF- $\beta$ 1 or components of the IGF2R-uPAR complex may contribute to the overall shape of the villous tree and thus influence functional capacity and pregnancy outcome.

*Chapter 4 : Acute effects of maternal  
IGF treatment on placental transport  
and the partitioning of substrates  
between the mother and fetus*

## 4.1 Introduction

Intrauterine growth restriction (IUGR) is an important obstetric and neonatal condition that affects approximately 6% of Australian births (Laws and Sullivan 2004). It is associated with increased fetal and neonatal mortality and morbidity, and neurological handicap in children (Fitzhardinge 1985; Kramer 1990; Kramer, Olivier *et al.* 1990; Low, Handley-Derry *et al.* 1992; Smith, Smith *et al.* 1998). Furthermore, IUGR is also associated with increased risk of poor health in offspring in adult life, including atherosclerosis (Martyn, Gale *et al.* 1998), coronary heart disease (Barker 1995), hypertension (Barker, Bull *et al.* 1990), insulin resistance, non-insulin dependent diabetes (Barker 1998; Barker and Clark 1997) and elevated plasma cholesterol (Barker, Martyn *et al.* 1993) in the offspring later in adult life. All of these are major public health concerns as they significantly contribute to the premature death and poor health of individuals in the Western world. Despite extensive investigations, the precise pathological events responsible for the development of IUGR have not been elucidated and therefore, currently there are no effective preventative treatments. A better understanding of the regulation of fetal growth may provide insight into the pathophysiology of IUGR, which is important not only for reproduction but for the long-term programming of adult health.

Fetal growth is dependent on maternal supply of nutrients and oxygen. To ensure a continuous delivery of nutrients to the fetus despite intermittent food intake, the maternal body undergoes several physiological changes during pregnancy which, in turn, are influenced by maternal factors including diet and hormonal milieu (Butte 2005; Owens 1991). The placenta is critical to these processes, as it forms the functional interface between the maternal and fetal circulations. It mediates all interactions between the mother and fetus, including transport of nutrients and oxygen from the mother to the fetus. The placenta may also indirectly influence the supply of substrate to the fetus through its elaboration of peptide and steroid hormones, secreted into the maternal and fetal circulations, which influence metabolism and alter nutrient utilization and substrate partitioning between the mother and fetus (Butte 2000; Catalano 1999; Ryan and Enns 1988). Thus, the placenta has the unique ability to alter the pattern of fetal development and affect pregnancy outcome.

The syncytiotrophoblast is the transporting epithelium of the placenta and maternal to fetal exchange of substrate is mediated by transporter systems embedded in the maternal facing, microvillous membrane (MVM) and the fetal facing, basal membrane (BM). Glucose and amino acids are the main substrates utilized by the fetus and thus it is not surprising that human IUGR fetuses display reduced plasma concentrations of both these substrates (Cetin, Corbetta *et al.* 1990; Cetin, Radaelli *et al.* 2001;

Economides and Nicolaides 1989) and significant reductions in placental nutrient transport capacity (Jansson and Powell 2006). For instance, System A sodium-dependent transporters are responsible for mediating the uptake and transfer of neutral amino acids, including alanine, glycine, glutamine and serine (McGivan and Pastor-Anglada 1994). The activities of System A transporters are reduced in IUGR pregnancies and correlate with severity of growth restriction (Glazier, Cetin *et al.* 1997; Jansson, Ylven *et al.* 2002; Mahendran, Donnai *et al.* 1993), suggesting that alterations in placental transport capacity represent an important mechanism for influencing/regulating fetal growth. Importantly, these studies also demonstrate that System A transporter activity perturbations were localised specifically to the syncytiotrophoblast MVM, and not BM. This implies that factors in the maternal circulation perfusing the placenta, have the potential to alter placental amino acid uptake and transfer through regulation of System A transporters on MVM.

Transport of glucose by the placenta occurs by facilitated carrier diffusion, primarily through glucose transporter (GLUT)-1. GLUT-1 is primarily localised to MVM (Jansson, Wennergren *et al.* 1993; Tadokoro, Yoshimoto *et al.* 1996; Takata, Kasahara *et al.* 1992), suggesting that placental glucose delivery across the BM is the rate-limiting step (Illsley 2000). In contrast to System A amino acid transport, and despite fetal hypoglycaemia, glucose transport activity is unaltered in placenta from human IUGR pregnancies (Jansson, Wennergren *et al.* 1993; Jansson, Ylven *et al.* 2002; Kainulainen, Jarvinen *et al.* 1997). Interestingly, the capacity for the placenta to deliver glucose is increased in BM of placenta from pregnancies complicated by insulin-dependent diabetes mellitus associated with increased fetal growth (Gaither, Quraishi *et al.* 1999; Jansson, Wennergren *et al.* 1999), but is unaltered in gestational diabetes, which is also associated with accelerated growth of the fetus (Jansson, Ekstrand *et al.* 2001). This work suggests that, glucose transporters are responsive to stimulation in early pregnancy and affect subsequent fetal growth. Further studies on the regulation of placental metabolism and maternal and fetal utilization of substrate may help to elucidate the mechanisms involved in placental transfer in pregnancies associated with fetal growth restriction. Certainly, factors on either side of the placenta may have impacts on placental metabolism and nutrient transfer and partitioning and thus, regulate fetal growth.

The insulin-like growth factors (IGFs) may be such factors, as they are abundantly expressed by the fetus and mother in most species (Fowden 2003; Murphy, Smith *et al.* 2006), with IGF-II also copiously synthesized by the placenta (Han and Carter 2000) Both IGFs play dominant roles in the regulation of fetal growth (Baker, Liu *et al.* 1993; DeChiara, Efstratiadis *et al.* 1990; Liu, Baker *et al.* 1993) and may operate via autocrine, paracrine and endocrine influences on placental transport capacity and nutrient



partitioning during pregnancy. IGF-I and IGF-II are anabolic polypeptides, expressed in most tissues, and circulate around the body in association with IGF-binding proteins (IGFBPs). IGFBPs affect the bioavailability of IGF-I and IGF-II (Jones and Clemmons 1995) and are considered to regulate the endocrine actions of IGFs, as they localise IGFs to specific cell types and modulate both IGF binding to receptors and growth promoting actions (Kelley, Oh *et al.* 1996; Wetterau, Moore *et al.* 1999). IGFs can bind to two types of IGF receptor, IGF1R and IGF2R, as well as the insulin receptor (InsR) with different affinities and effects. During pregnancy, substantial changes in the abundance of IGFs and IGFBPs in the maternal circulation occur (Donovan, Giudice *et al.* 1991; Gargosky, Moyses *et al.* 1990; Hills, English *et al.* 1996; Nason, Binder *et al.* 1996; Sohlstrom, Katsman *et al.* 1998b; Travers, Madon *et al.* 1990; Wilson, Bennett *et al.* 1982), which further suggest an important regulatory role for the IGFs in pregnancy.

Studies performed *in vitro* in human placental trophoblasts have demonstrated that IGFs have the ability to influence glucose and amino acid transport across the placenta (Bloxam, Bax *et al.* 1994; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998). These may have been due to direct impacts on nutrient transporter expression. Certainly, IGF-I induces gene and protein expression of *Slc2a1* and *Slc2a3* (genes that encode GLUT-1 and GLUT-3) in bovine chromaffin cells, *in vitro* (Fladeby, Skar *et al.* 2003) and equine articular chondrocytes (Phillips, Ferraz *et al.* 2005). Exogenous IGF-I also promotes the re-distribution/translocation of GLUT-1, GLUT-3 and GLUT-4 from their intracellular storage sites, to the cell surface in muscle cell lines, which resulted in increased glucose uptake and transfer (Bilan, Mitsumoto *et al.* 1992a; Bilan, Mitsumoto *et al.* 1992b; Wilson, Mitsumoto *et al.* 1995). Interestingly, in mice where the *Igf2 P0* transcript, responsible for placental IGF-II synthesis, has been deleted, System A amino acid uptake by the placenta was up-regulated in early pregnancy (Constancia, Hemberger *et al.* 2002) and placental glucose transport enhanced throughout gestation (Constancia, Angiolini *et al.* 2005). This may have been an adaptive mechanism to increase fetal growth in spite of reduced development and passive permeability of the placenta in these mice (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004).

IGFs appear to also modify placental substrate transfer through impacts on placental structure. Certainly, diminished circulating concentrations of both IGFs in undernourished guinea pig mothers (Roberts, Kind *et al.* 2002; Roberts, Sohlstrom *et al.* 2001a; Roberts, Sohlstrom *et al.* 2001b; Sohlstrom, Katsman *et al.* 1998b) or reduced placental synthesis of IGF-II in *Igf2 P0* mice (Constancia, Hemberger *et al.* 2002), increased thickness of the barrier for transport and reduced the surface area across which transport can occur. These alterations in placental development would be expected to reduce the

capacity of the placenta to deliver substrate to the fetus and may explain the development of fetal growth restriction. These studies indicate that placental transport alterations can contribute directly to the growth of the fetus and are likely to involve factors including IGF-I and IGF-II. In a more recent investigation undertaken in guinea pigs, maternal administration of IGF-I and IGF-II during early to mid pregnancy increased fetal and placental growth in mid gestation, with IGF-II also increasing maternal interscapular fat mass (Sohlstrom, Fernberg *et al.* 2001). It is not known whether the anabolic effects seen in response to IGF treatment are a consequence of altered placental nutrient transport and substrate partitioning between the mother and fetus.

The aim of this study was to investigate how IGF-I or IGF-II supplementation to the mother during early to mid pregnancy, exerts acute effects on maternal growth and body composition and fetal and placental growth. The acute effects of such a treatment on placental transfer of glucose and amino acids and nutrient utilization in the mother and fetus, were also examined. To do this, pregnant female guinea pigs were treated from day 20 of pregnancy (term ~70 days) with IGF-I, IGF-II (both 1 mg/kg/day) or vehicle. On day 35 of pregnancy, whilst the treatment was still occurring, transfer of the non-metabolisable radioanalogues [<sup>3</sup>H]-methyl-D-glucose (MG) and [<sup>14</sup>C]-amino-isobutyric acid (AIB) *in vivo* was measured, just prior to maternal death. Circulating fetal and maternal metabolites were quantitated and fetal and placental weights and maternal body composition were also recorded. This project was part of a shared cohort of animals used for Prue Standen's honours work analyses.

## 4.2 Results

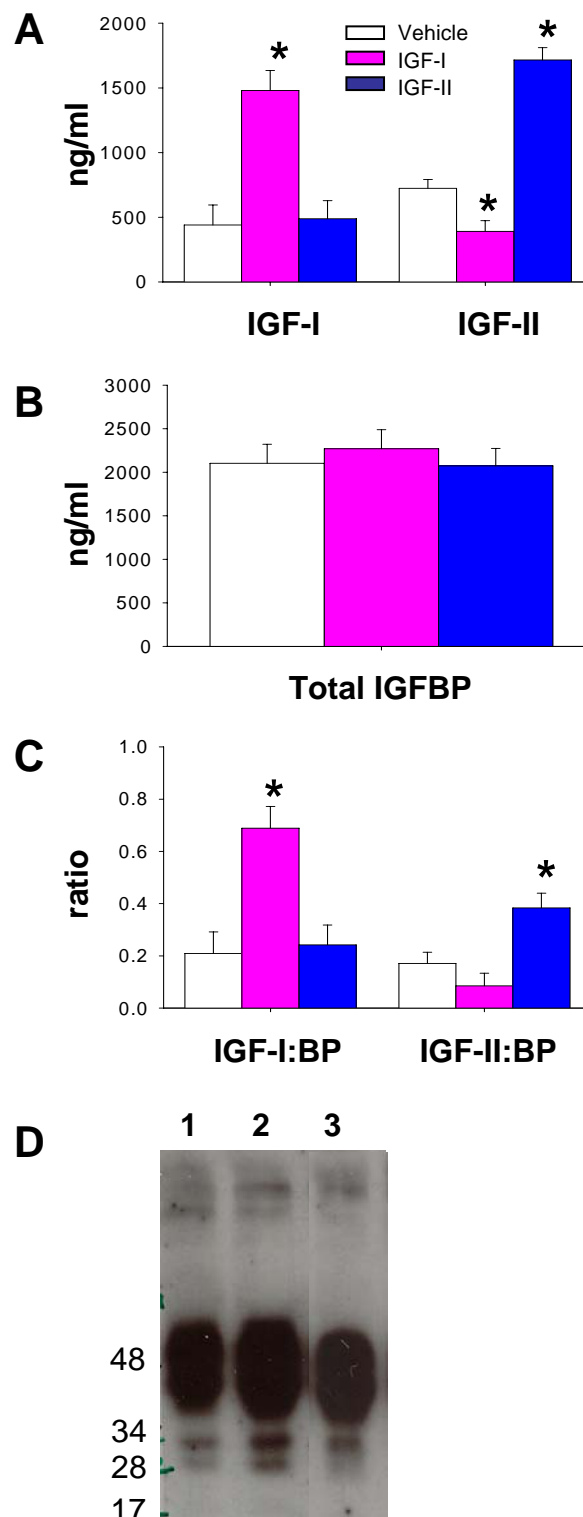
### *4.2.1.1 Acute effect of maternal IGF treatment on the circulating concentration of IGFs and IGFBPs*

To determine the concentration of IGFs we achieved in the maternal circulation in response to this treatment, size exclusion HPLC (at pH 2.5) and specific RIAs were performed on maternal plasma. Exogenous IGF-I increased maternal plasma IGF-I by 340% ( $P = 0.001$ ) and reduced that of IGF-II by 45% ( $P = 0.008$ , Figure 4.1a). Exogenous IGF-II did not alter plasma IGF-I concentrations but increased plasma IGF-II by 240% ( $P = 0.004$ , Figure 4.1a). Total apparent IGFBP activity in maternal plasma was not altered by exogenous maternal IGF (Figure 4.1b). Maternal IGF-I treatment increased the ratio of IGF-I to IGFBPs in plasma by 230% ( $P = 0.004$ ), while IGF-II increased the ratio of IGF-II to IGFBPs in plasma by 125% ( $P = 0.04$ , Figure 4.1c).

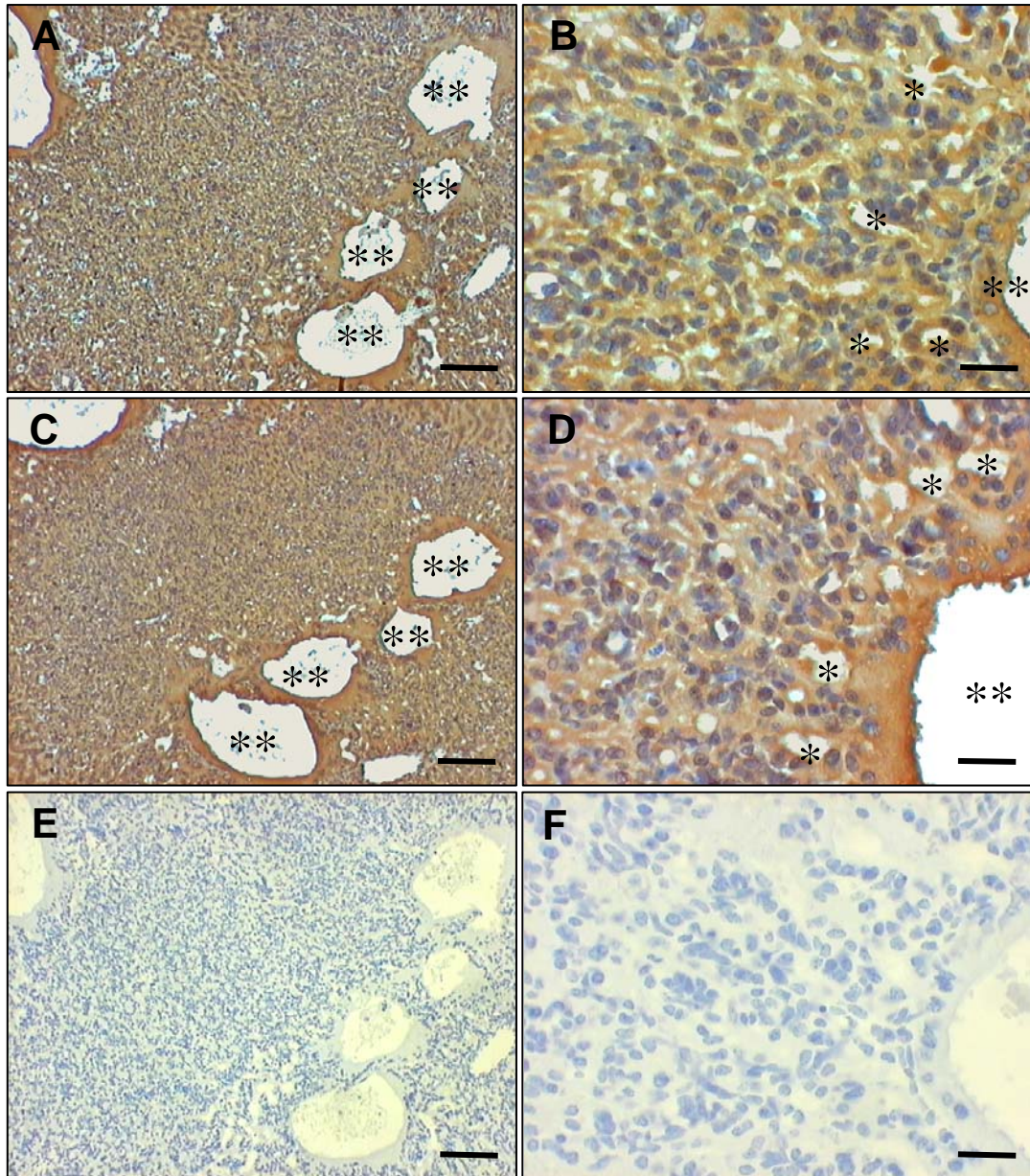
Western-ligand blot analysis of pooled maternal plasma samples (Figure 4.1d) revealed distinct ~46-kDa, ~30-kDa and ~28-kDa bands, which were identified as IGFBP-3, IGFBP-2 and IGFBP-1, respectively, on the basis of their apparent molecular mass. Additionally, a faint band corresponding to 17kDa was seen, which was identified as IGFBP-4. IGFBP-3 was the most abundant binding protein in all treatment groups, followed by IGFBP-2, -1 and -4 (Figure 4.1d). IGF-I treatment increased the plasma levels of IGFBP-3, -2 and -1. IGF-II treatment did not alter plasma IGFBP-3 concentration, but it increased plasma IGFBP-2 concentration and reduced that of IGFBP-1 (Figure 4.1d).

### *4.2.1.2 IGF receptor protein localization in the guinea pig placenta on day 35 of pregnancy*

To establish that IGF1R and IGF2R are expressed by the guinea pig placenta during the IGF treatment, immuno-labelling was performed on guinea pig placenta recovered from vehicle treated mothers killed on day 35 of pregnancy (Figure 4.2). IGF1R and IGF2R were ubiquitously expressed by the guinea pig placenta, with profuse cytoplasmic staining observed in trophoblast and fetal endothelium of the labyrinth and trophoblast of the interlobium (Figure 4.2a, c). Both IGF receptor proteins were concentrated on the apical surface of trophoblast lining large maternal blood spaces (Figure 4.2b, d).



**Figure 4.1** Acute effect of exogenous maternal IGF in early to mid pregnancy on plasma IGF-I, IGF-II and IGFBP concentrations in the mother. The effect of exogenous maternal IGFs on maternal circulating IGF-I, IGF-II (a) and total IGFBP concentrations (b) and bioavailability of IGFs in the circulation indicated by IGF to IGFBP ratios (c) during treatment on day 35 of pregnancy, as determined by HPLC and RIAs. Western ligand blot of IGFBPs in pooled maternal plasma (d) from; vehicle (*lane 1*), IGF-I (*lane 2*) and IGF-II (*lane 3*) treated animals on day 35 of gestation with molecular masses (kDa) shown (*left of blot*). Data are from 3-9 mothers per treatment and values are expressed as means  $\pm$  SEM. Asterisks denote statistically significant difference compared to the vehicle group,  $P < 0.034$ , determined by general linear model univariate ANOVA with Bonferroni Post Hoc Tests.



**Figure 4.2 Localisation of IGF receptors in the guinea pig placenta in mid-pregnancy.** Representative mid-sagittal serial sections of placenta on day 35 of pregnancy immuno-labelled for the type 1 (a and b) and type 2 (c and d) IGF receptors. Representative negative control placental section displayed (e and f). Two asterisks indicate maternal blood sinusoids and single asterisks indicate maternal blood spaces. Scale bars = 400µm (a, c, e) and 40µm (b, d, f).

#### *4.2.1.3 Acute effect of maternal IGF treatment on maternal body composition*

Maternal exogenous IGF-I and IGF-II did not affect maternal weight gain over the time of treatment in mid-pregnancy (data not shown). Maternal IGF-I treatment increased the absolute and relative weights of the maternal kidneys (+16%,  $P < 0.001$  and +15%,  $P = 0.024$ , respectively) and spleen (+40%,  $P = 0.005$  and +38%,  $P = 0.038$ ), compared to vehicle (Table 4.1). Maternal IGF-II elevated the absolute weight of the heart (+13%,  $P = 0.58$ ), however its fractional weight was unaltered (Table 4.1)

#### *4.2.1.4 Acute effect of maternal IGF treatment on litter composition, fetal growth and placental weight*

Maternal treatment with IGF-I or IGF-II did not alter litter size or composition on day 35 of pregnancy (Figure 4.3a). Maternal IGF-I supplementation increased the weights of the fetus (+11%,  $P = 0.048$ ) and placenta (+13%,  $P = 0.036$ ) compared to vehicle (Figure 4.3 b, c). IGF-II treatment of the mother did not alter placental or fetal weights. There was no acute effect of maternal IGF supplementation on the fetal weight to placental weight ratio (data not shown).

#### *4.2.1.5 Acute effect of maternal IGF treatment on uptake of methyl glucose and amino isobutyric acid by the placenta*

Treating the mother with IGF-I during early to mid pregnancy increased total placental MG uptake (+42%,  $P = 0.036$ ) and AIB uptake per gram and total placenta (+51%,  $P = 0.003$  and +68%,  $P = 0.005$ , respectively), compared to vehicle treated mothers in mid-gestation (Figure 4.4). Placenta from mothers treated with IGF-I had higher total AIB uptake compared with those treated with IGF-II (+61%,  $P = 0.014$ ) (Figure 4.4).

#### *4.2.1.6 Acute effect of maternal IGF treatment on uptake of methyl glucose and amino isobutyric acid by the fetus*

Treating the mother with IGF-I during early to mid pregnancy increased per gram and total fetal MG content (+48%,  $P = 0.012$  and +59%,  $P = 0.004$ , respectively) and per gram and total fetal AIB uptake (+68%,  $P = 0.005$  and +90%,  $P = 0.004$ , respectively), compared to vehicle treated mothers in mid-gestation (Figure 4.5). There was no acute effect of exogenous maternal IGF-II on uptake of either radio-labelled substrate by the fetus.

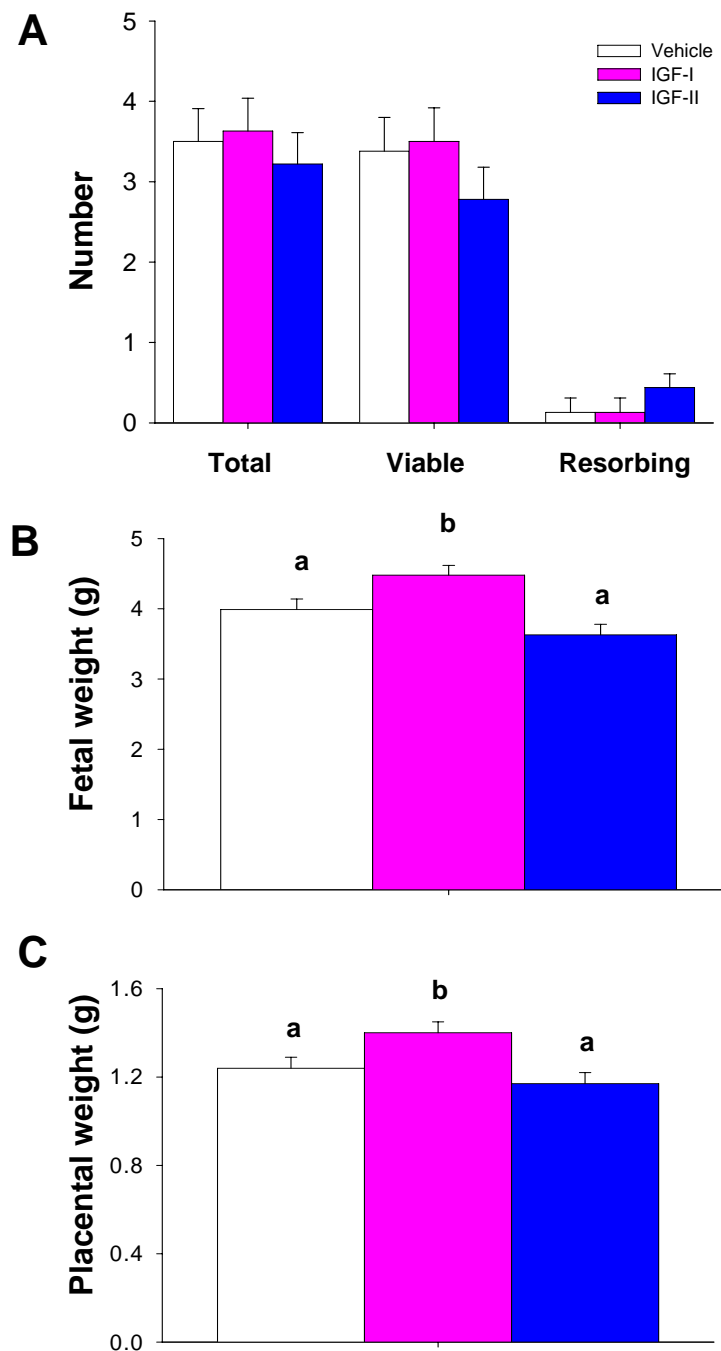
Table 4.1 Acute effect of maternal IGF treatment in early to mid pregnancy on maternal body composition

	Vehicle	IGF-I	IGF-II
Number of dams	8	9	9
Weight at d35	747 ± 16	788 ± 15	753 ± 15
Net body mass**	700 ± 30	735 ± 23	688 ± 31
Uterus* (g)	47 ± 5	53 ± 5	37 ± 5
Adrenals (g)	0.33 ± 0.02	0.35 ± 0.01	0.36 ± 0.01
(% Body weight)†	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
Kidneys (g)	4.54 ± 0.1 <sup>a</sup>	5.28 ± 0.1 <sup>b</sup>	4.84 ± 0.1 <sup>ab</sup>
(% Body weight)†	6.5 ± 0.03 <sup>a</sup>	7.5 ± 0.02 <sup>b</sup>	6.8 ± 0.02 <sup>a</sup>
Spleen (g)	1.51 ± 0.1 <sup>a</sup>	2.11 ± 0.1 <sup>b</sup>	1.57 ± 0.1 <sup>ab</sup>
(% Body weight)†	0.21 ± 0.02 <sup>a</sup>	0.29 ± 0.02 <sup>a</sup>	0.22 ± 0.02 <sup>a</sup>
Pancreas (g)	1.98 ± 0.2	2.13 ± 0.2	2.07 ± 0.2
(% Body weight)†	0.28 ± 0.03	0.29 ± 0.03	0.29 ± 0.03
Liver (g)	25.5 ± 1.0	26.6 ± 0.9	25.1 ± 0.9
(% Body weight)†	3.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
Lung (g)	4.21 ± 0.3	3.93 ± 0.3	3.98 ± 0.3
(% Body weight)†	0.60 ± 0.04	0.54 ± 0.03	0.56 ± 0.03
Brain (g)	4.20 ± 0.4	4.32 ± 0.4	4.65 ± 0.3
(% Body weight)†	0.60 ± 0.05	0.59 ± 0.05	0.66 ± 0.05
Heart (g)	2.01 ± 0.1 <sup>a</sup>	2.19 ± 0.1 <sup>ac</sup>	2.28 ± 0.1 <sup>c</sup>
(% Body weight)†	0.30 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
Ovaries (g)	0.17 ± 0.08	0.16 ± 0.07	0.26 ± 0.07
(% Body weight)†	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
Stomach (g)	7.14 ± 0.6	7.04 ± 0.6	6.55 ± 0.6
(% Body weight)†	1.4 ± 0.2	0.97 ± 0.2	0.85 ± 0.2
S. intestine (g)	15.3 ± 1.3	15.3 ± 1.3	15.9 ± 1.3
(% Body weight)†	2.4 ± 0.2	1.7 ± 0.2	2.2 ± 0.2
L. intestine (g)	7.99 ± 0.8	9.95 ± 0.8	7.31 ± 0.8
(% Body weight)†	1.1 ± 0.2	1.7 ± 0.2	1.0 ± 0.2

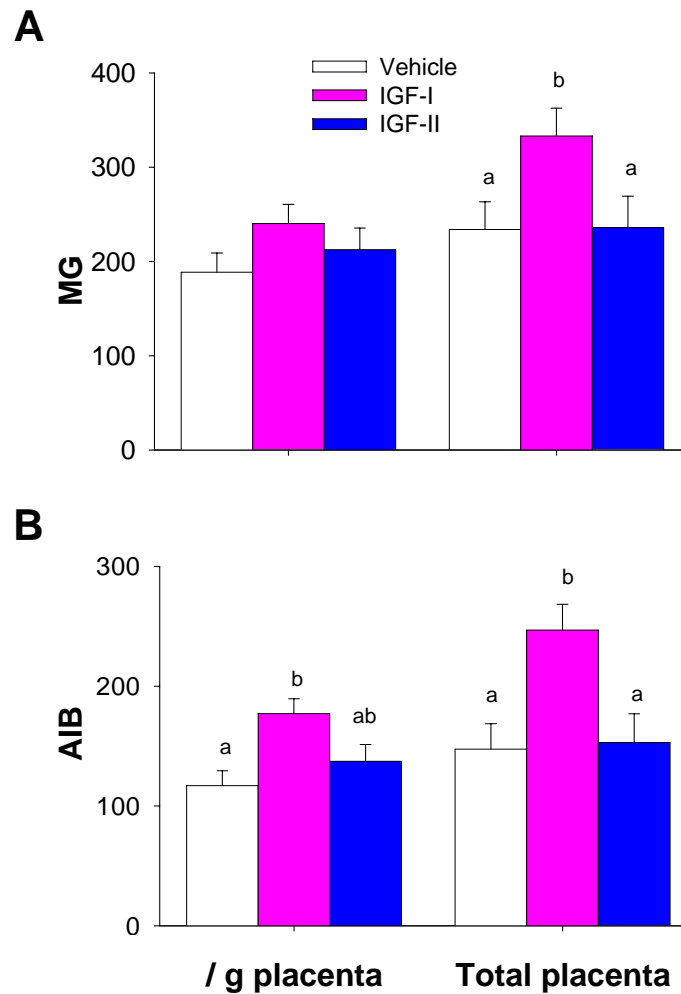
Caecum (g)	10.2 ± 0.5	10.6 ± 0.5	10.3 ± 0.5
(% Body weight) <sup>‡</sup>	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Total GIT (g)	45.0 ± 1.6	43.0 ± 1.6	40.0 ± 1.6
(% Body weight) <sup>‡</sup>	6.2 ± 0.2	5.8 ± 0.3	5.5 ± 0.2
M.soleus (g)	0.17 ± 0.02	0.20 ± 0.2	0.16 ± 0.02
(% Body weight) <sup>‡</sup>	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
M.biceps (g)	0.42 ± 0.01	0.43 ± 0.01	0.40 ± 0.01
(% Body weight) <sup>‡</sup>	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
M.triceps (g)	2.13 ± 0.1	2.26 ± 0.1	2.09 ± 0.1
(% Body weight) <sup>‡</sup>	0.30 ± 0.01	0.31 ± 0.01	0.29 ± 0.01
M.gastroc (g)	2.13 ± 0.1	2.04 ± 0.1	2.09 ± 0.1
(% Body weight) <sup>‡</sup>	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01
Perirenal fat (g)	5.3 ± 0.5	5.72 ± 0.5	6.3 ± 0.5
(% Body weight) <sup>‡</sup>	0.74 ± 0.07	0.79 ± 0.07	0.87 ± 0.07
Retro fat (g)	7.65 ± 0.5	7.95 ± 0.5	7.90 ± 0.5
(% Body weight) <sup>‡</sup>	1.2 ± 0.1	0.85 ± 0.1	1.2 ± 0.06
Inter fat (g)	9.73 ± 0.8	10.2 ± 0.8	9.36 ± 0.8
(% Body weight) <sup>‡</sup>	1.1 ± 0.06	1.1 ± 0.05	1.1 ± 0.05
Skin (g)	109 ± 5	122 ± 5	118 ± 5
(% Body weight) <sup>‡</sup>	15 ± 0.6	17 ± 0.6	17 ± 0.6
Carcass (g)	382 ± 8	397 ± 8	389 ± 8
(% Body weight) <sup>‡</sup>	54 ± 0.8	54 ± 0.8	55 ± 0.8

Data expressed as means ± SEM. \*Uterus and contents. \*\*Net body mass is weight at post-mortem minus the uterus and contents. †Tissue weight calculated as a percentage of net body mass. GIT, gastrointestinal tract; Inter fat, interscapular fat; L. intestine, large intestine; M.gastroc, M.gastrocnemius; Retro fat, retroperitoneal fat; S. intestine, small intestine. Different superscripts denote difference between groups, a vs b, P < 0.05 (significant), a vs c P = 0.058 (not significant), as determined by General Linear Univariate ANOVA with Bonferoni Post Hoc Tests.

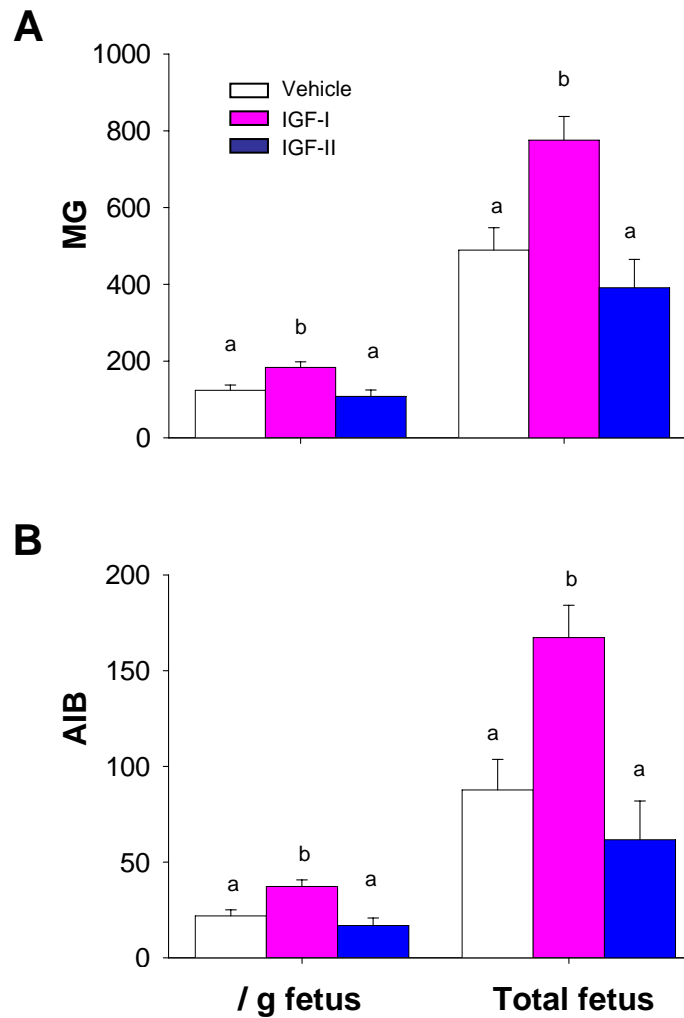




**Figure 4.3** Acute effect of maternal IGF treatment in early to mid pregnancy on litter composition and fetal and placental weights. Mothers treated from day 20 of pregnancy with vehicle, IGF-I or IGF-II, were killed during the treatment on day 35 of pregnancy. Total litter size, viable and resorbing fetuses were enumerated (a) and fetal (b) and placental weights (c) were recorded. Data represents the mean  $\pm$  SEM (a) and estimated marginal mean  $\pm$  SEM, controlled for the number of viable pups per litter (b, c). Different superscripts denote statistical significance difference between groups as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject and the fetus as a repeated measure; a vs b,  $p < 0.05$ . The number of implantation sites and mothers per group are vehicle: 27 and 8, respectively, IGF-I: 31 and 8, respectively and IGF-II: 20 and 9, respectively.



**Figure 4.4** Acute effect of maternal IGF treatment in early to mid pregnancy on placental uptake of substrate. Placental uptake of [ $^3\text{H}$ ]-methyl-D-glucose (MG, a) and [ $^{14}\text{C}$ ]-aminoisobutyric acid (AIB, b). Data are expressed as estimated marginal mean DPM  $\pm$  SEM, controlling for the number of viable pups per litter. Different superscripts denote statistically significant difference between groups as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject and the fetus as a repeated measure, a vs b,  $p < 0.05$ . The number of placenta and mothers per group are vehicle: 27 and 8, respectively, IGF-I: 31 and 8, respectively and IGF-II: 20 and 9, respectively.



**Figure 4.5** Acute effect of maternal IGF treatment in early to mid pregnancy on fetal utilization of substrate. Fetal uptake of [<sup>3</sup>H]-methyl-D-glucose (MG, a) and [<sup>14</sup>C]-aminoisobutyric acid (AIB, b). Data are expressed as estimated marginal mean DPM ± SEM, controlling for the number of viable pups per litter. Different superscripts denote statistically significant difference between groups as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject and the fetus as a repeated measure, a vs b,  $p < 0.05$ . The number of fetuses and mothers per group are vehicle: 27 and 8, respectively, IGF-I: 31 and 8, respectively and IGF-II: 20 and 9, respectively.

#### *4.2.1.7 Acute effect of maternal IGF treatment on circulating methyl glucose and amino isobutyric acid in the mother*

There was no acute effect of maternal IGF treatment on the circulating concentrations of MG or AIB in the mother (Figure 4.6a).

#### *4.2.1.8 Acute effect of maternal IGF treatment on uptake of methyl glucose by the mother*

Uptake of methyl glucose by individual maternal organs and tissues are displayed in Table 4.2. When tissues were analysed individually, there was no significant effect of maternal IGF treatment on the uptake of MG per gram of tissue (Table 4.2). However, maternal IGF-I supplementation increased total spleen uptake of MG compared with vehicle and IGF-II treated mothers (+97%,  $P = 0.012$ , +65%,  $P = 0.043$ , respectively).

When all tissues were combined, there was no acute effect of maternal IGF treatment on MG uptake per gram of tissue (Figure 4.6b). However, IGF-I increased total uptake of MG by muscle (+23%,  $P = 0.015$ ) (Figure 4.6 c).

#### *4.2.1.9 Acute effect of maternal IGF treatment on uptake of amino isobutyric acid by the mother*

Uptake of amino isobutyric acid by individual maternal organs and tissues are displayed in Table 4.3. Exogenous IGF-I treatment acutely increased per gram and total AIB uptake by the maternal brain (+77%,  $P = 0.007$ ; +85%,  $P = 0.004$ , respectively) and spleen (+52%,  $P = 0.035$ ; +222%,  $P = 0.004$ , respectively) (Table 4.3). Uptake (DPM / g) of AIB by the maternal triceps was increased by maternal IGF-I and IGF-II (+35 and +40%, both  $P = 0.078$ ) and maternal IGF-I increased total uptake by maternal gastrocnemius (+29%,  $P = 0.07$ ) but these failed to reach statistical significance.

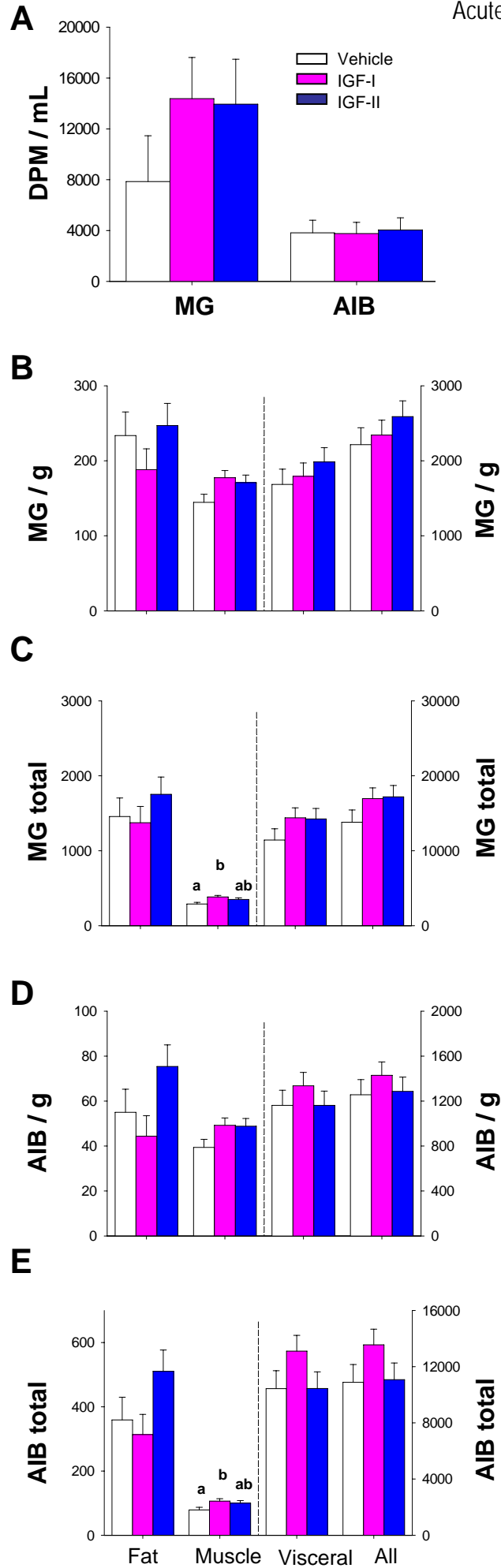
When all tissues were combined, there was no acute effect of maternal IGF treatment on AIB uptake per gram of tissue (Figure 4.6d). However, IGF-I increased total uptake of AIB by muscle (+35%,  $P = 0.05$ ) (Figure 4.6e).

**Table 4.2 Acute effect of maternal IGF treatment in early to mid pregnancy on methyl glucose uptake in the mother**

		Vehicle	IGF-I	IGF-II
Number		7	9	9
Brain	(DPM / gram)	151 ± 21	182 ± 17	194 ± 19
	(total DPM)	632 ± 104	792 ± 93	896 ± 94
Heart	(DPM / gram)	334 ± 69	228 ± 68	274 ± 62
	(total DPM)	673 ± 143	490 ± 127	629 ± 128
Liver	(DPM / gram)	269 ± 38	338 ± 34	312 ± 34
	(total DPM)	6653 ± 1004	8894 ± 895	7940 ± 903
Lungs	(DPM / gram)	595 ± 150	548 ± 128	507 ± 123
	(total DPM)	2165 ± 536	2123 ± 458	1988 ± 440
Kidneys	(DPM / gram)	442 ± 125	470 ± 117	698 ± 120
	(total DPM)	1962 ± 639	2472 ± 597	3492 ± 616
Spleen	(DPM / gram)	166 ± 25 <sup>a</sup>	226 ± 23 <sup>b</sup>	178 ± 23 <sup>ab</sup>
	(total DPM)	238 ± 53 <sup>a</sup>	469 ± 48 <sup>b</sup>	285 ± 48 <sup>a</sup>
Triceps	(DPM / gram)	73 ± 7	93 ± 7	89 ± 7
	(total DPM)	155 ± 18	209 ± 16	189 ± 16
Gastroc.	(DPM / gram)	71 ± 6	85 ± 6	83 ± 6
	(total DPM)	134 ± 13	175 ± 12	156 ± 12
Interscap.	(DPM / gram)	43 ± 3	41 ± 3	42 ± 3
	(total DPM)	392 ± 42	420 ± 37	401 ± 37
Perirenal	(DPM / gram)	112 ± 22	84 ± 20	103 ± 20
	(total DPM)	532 ± 95	450 ± 84	576 ± 85
Retroperit.	(DPM / gram)	65 ± 26	70 ± 21	88 ± 21
	(total DPM)	459 ± 212	553 ± 175	693 ± 177

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Different superscripts denote difference between groups, a vs b,  $P < 0.05$  (significant); a vs c  $P = 0.07$  (not significant), as determined by General Linear Univariate ANOVA with Bonferoni Post Hoc Tests.

**Figure 4.6 Acute effect of maternal IGF treatment in early to mid pregnancy on maternal utilization of substrate.** Plasma (a) and tissue content of [<sup>3</sup>H]-methyl-D-glucose, (MG, b and c) and [<sup>14</sup>C]-aminoisobutyric acid, (AIB, d and e). Uptake expressed per gram (b and d) of tissue or total (c and e). Values are expressed as mean DPM ± SEM from 8-9 mothers per treatment. Different superscripts denote statistically significant difference between groups determined by Univariate ANOVA with Bonferoni Post Hoc Tests; a vs b, p < 0.05.



**Table 4.3 Acute effect of maternal IGF treatment in early to mid pregnancy on amino isobutyric acid uptake in the mother**

		Vehicle	IGF-I	IGF-II
Number		7	9	9
Brain	(DPM / gram)	3.1 ± 0.5 <sup>a</sup>	5.5 ± 0.5 <sup>b</sup>	4.5 ± 0.5 <sup>a</sup>
	(total DPM)	13 ± 2 <sup>a</sup>	24 ± 2 <sup>b</sup>	20 ± 2 <sup>ab</sup>
Heart	(DPM / gram)	104 ± 16	78 ± 14	91 ± 14
	(total DPM)	207 ± 33	168 ± 29	205 ± 30
Liver	(DPM / gram)	296 ± 67	354 ± 59	293 ± 60
	(total DPM)	6951 ± 1427	9114 ± 1271	7271 ± 1282
Lungs	(DPM / gram)	338 ± 130	403 ± 111	282 ± 107
	(total DPM)	1217 ± 485	1568 ± 415	1104 ± 398
Kidneys	(DPM / gram)	229 ± 36	247 ± 34	244 ± 35
	(total DPM)	1962 ± 639	2472 ± 597	3492 ± 616
Spleen	(DPM / gram)	160 ± 22 <sup>a</sup>	243 ± 20 <sup>b</sup>	182 ± 20 <sup>a</sup>
	(total DPM)	228 ± 56 <sup>a</sup>	507 ± 50 <sup>b</sup>	307 ± 50 <sup>a</sup>
Triceps	(DPM / gram)	20 ± 2 <sup>a</sup>	27 ± 2 <sup>c</sup>	28 ± 2 <sup>c</sup>
	(total DPM)	44 ± 6	61 ± 6	58 ± 6
Gastroc.	(DPM / gram)	19 ± 2	22 ± 2	21 ± 2
	(total DPM)	35 ± 3 <sup>a</sup>	45 ± 3 <sup>c</sup>	39 ± 3 <sup>ac</sup>
Interscap.	(DPM / gram)	11 ± 1	10 ± 1	11 ± 1
	(total DPM)	104 ± 13	104 ± 11	108 ± 11
Perirenal	(DPM / gram)	26 ± 10	23 ± 9	40 ± 9
	(total DPM)	136 ± 52	125 ± 46	216 ± 47
Retroperit.	(DPM / gram)	16 ± 5	12 ± 4	21 ± 4
	(total DPM)	114 ± 40	96 ± 32	171 ± 33

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Different superscripts denote difference between groups, a vs b,  $P < 0.05$  (significant) and a vs c  $P < 0.078$  (not significant), as determined by General Linear Univariate ANOVA with Bonferoni Post Hoc Tests.

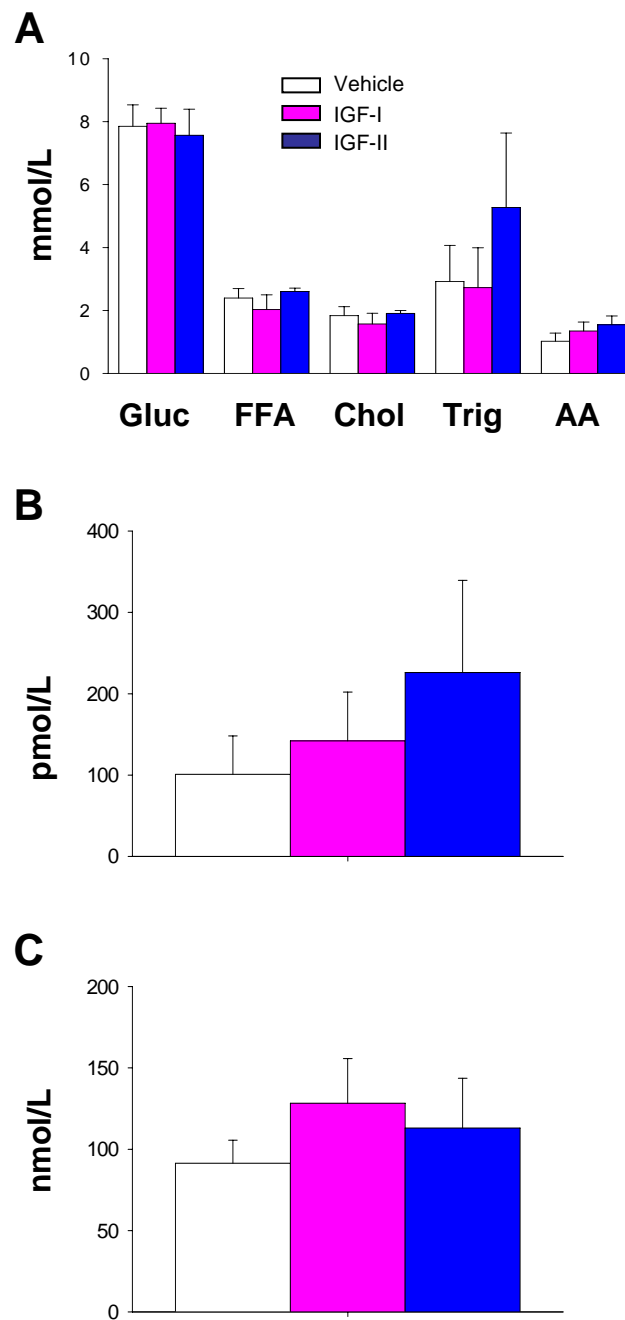


*4.2.1.10 Acute effect of maternal IGF treatment on circulating metabolites in the mother*

There was no acute effect of maternal IGF treatment on the circulating concentrations of glucose, free fatty acids, amino acids, triglycerides or cholesterol in the mother (Figure 4.7a), as determined by enzymatic and colorimetric assays. There were no significant correlations of maternal circulating metabolite concentrations with maternal body composition nor with fetal or placental weights (data not shown).

*4.2.1.11 Acute effect of maternal IGF treatment on circulating hormones in the mother*

To determine whether treatment of the mother during early to mid pregnancy with IGFs altered maternal circulating estradiol (Figure 4.7b) and progesterone (Figure 4.7c), their concentrations were determined using RIAs. There was a trend for increased circulating estradiol (+150%) and progesterone concentrations (+53%), in mid pregnancy following exogenous maternal IGF-II, however these also did not reach statistical significance ( $P = 0.08$ ). There were no significant correlations of maternal circulating hormone concentrations with maternal body composition or circulating metabolite concentrations or with fetal or placental weights (data not shown).



**Figure 4.7** Acute effect of maternal IGF treatment **in early to mid pregnancy** on maternal circulating metabolite and hormone concentrations. Plasma concentrations of amino acids (AA), cholesterol (Chol), free fatty acids (FFA), glucose (Gluc) and triglycerides (Trig) (a), as well as estradiol (b) and progesterone (c) in the mother, were determined. Data are from 6-8 mothers per treatment and values are expressed as mean  $\pm$  SEM.

### 4.3 Discussion

The results of the current study indicate that maternal IGF-I treatment from early pregnancy, increases placental glucose and System A amino acid transfer and fetal uptake, while enhancing maternal muscle substrate utilization in mid pregnancy. Furthermore, maternal administration of IGF-I also increased placental and fetal growth and altered maternal body composition. By contrast, IGF-II treatment did not affect mid-gestational placental glucose and System A amino acid transport and substrate partitioning between the mother and fetus. IGF-II also did not alter fetal, placental nor maternal growth in the current study, despite increased fetal and placental growth a few days following treatment in a similar study previously performed in guinea pigs (Sohlstrom, Fernberg *et al.* 2001).

It is well established that human pregnancies complicated by IUGR display altered activity and/or expression of placental nutrient and ion transporters (Jansson and Powell 2000; Jansson and Powell 2006). In the current study, exogenous maternal IGF-I increased the activity of the System A amino acid transporters in mid pregnancy, as measured by AIB uptake per gram and total placental weight, and fetal AIB content. Indeed, System A transporter activity on the MVM of the syncytiotrophoblast is reduced in human placenta from IUGR pregnancies (Glazier, Cetin *et al.* 1997; Jansson, Ylven *et al.* 2002; Mahendran, Donnai *et al.* 1993) and down-regulated in IUGR induced by maternal protein restriction in rats (Malandro, Beveridge *et al.* 1996) and uterine artery ligation in guinea pigs (Jansson and Persson 1990). Additionally, there is a positive association of System A transport and fetal growth in human pregnancy (Dicke and Henderson 1988; Mahendran, Donnai *et al.* 1993). Furthermore, partial inhibition of System A activity by administration of meAIB to pregnant rats results in IUGR (Cramer, Beveridge *et al.* 2002), clearly highlighting the importance of system A transport functionality for fetal growth. Work performed in rats more recently has demonstrated that, reductions in System A activity precede the development of IUGR (Jansson, Pettersson *et al.* 2006), suggesting that alterations in placental transport are the cause of alterations in fetal growth. Indeed in the current study, elevated maternal plasma IGF-I also increased fetal and placental weights.

In addition to effects on amino acid transfer in the current study, elevated maternal IGF-I treatment increased overall placental uptake of glucose. Interestingly, IGF-I did not alter glucose uptake per gram of placenta, suggesting that placental glucose metabolism is unaltered and that increased total placental uptake is due to increased placental mass observed with the treatment. Nevertheless, placental supply of glucose to the fetus is increased as indicated by elevated MG concentrations in fetuses. Indeed, fetuses from IGF-I treated mothers appear to be more metabolically active as they utilized more glucose

and System A amino acids per gram of fetal tissue. This increase may have been related to indirect effects of maternal IGF-I on transplacental substrate transport, as amino acid and glucose are potent stimulators of insulin release from the fetal pancreas, which regulates fetal growth. Concomitantly, maternal exogenous IGF-I accelerated fetal growth on day 35 (current study), with both IGF-I and IGF-II increasing fetal weight on day 40 (Sohlstrom, Fernberg *et al.* 2001). This is consistent with reported reductions in maternal plasma IGF-I concentrations from 20 weeks to delivery in human pregnancies complicated by IUGR (Holmes, Montemagno *et al.* 1997).

The cellular mechanisms responsible for increased placental transport in IGF-I treated mothers remain to be established. It is hard to determine whether increased glucose and amino acid supply were caused by an alteration in the concentration gradient between the fetal and maternal circulations, which is known to influence substrate partitioning between the mother and fetus, as fetal plasma could not be recovered at this gestational age. However, this seems unlikely, as the circulating concentrations of amino acids and glucose, as well as other metabolites in the mother, were unaltered by either IGF treatment on day 35 (current study) or on day 40 of pregnancy (Sohlstrom, Fernberg *et al.* 2001). Instead, we speculate that maternal IGFs may have exerted an endocrine influence over the placenta, as we found abundant IGF1R and IGF2R immunoreactivity in trophoblast, particularly on the surface facing maternal blood spaces. If this is the case, maternal exogenous IGF-I may have increased placental transport by altering placental blood flow through its vasorelaxant effects, as previously demonstrated *in vitro* (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995) and *in vivo* (Schini-Kerth 1999; Yang, Chao *et al.* 2006).

Alternatively, IGF-I effects on placental substrate transfer may have been mediated through direct stimulation of transporter activity or expression, particularly in the case of System A amino acid transport, which was increased per gram of placenta. The System A amino acid transporters are encoded by the *Slc38* gene family and give rise to three isoforms, SNAT1, SNAT2 and SNAT4, which are expressed by the placenta of humans (Desforges, Lacey *et al.* 2006; Wang, Huang *et al.* 2000), rats (Mackenzie and Erickson 2004) and mice (Constancia, Angiolini *et al.* 2005). It was recently demonstrated that restriction of protein intake by pregnant rats, which reduced maternal circulating IGF-I, decreased SNAT2 protein expression and System A activity, resulting in IUGR (Jansson, Pettersson *et al.* 2006). Furthermore, IGF-I over-abundance in growth hormone transgenic mice, altered placental expression of other amino acid transporters (Matthews, Beveridge *et al.* 1999). Unfortunately, no study has characterized the expression of amino acid transporters in the guinea pig and thus, future

research endeavours should be directed at identifying them and determining the effect of IGF supplementation on their abundance and activity.

In addition to direct effects on placental transporter expression, IGF-I may enhance the function of the System A transporters, leading to increased uptake by the placenta and increased delivery of substrate (AIB) to the fetus. The function of the System A transporters depends on the activity of the placental sodium-proton exchanger and the sodium-potassium-ATPase pump, both of which are reduced on the MVM of placenta in pregnancies complicated by IUGR (Glazier, Cetin *et al.* 1997; Johansson, Glazier *et al.* 2002; Johansson, Karlsson *et al.* 2003). Insulin is known to stimulate the sodium-proton exchanger in red blood cells (Ceolotto, Conlin *et al.* 1997) and other tissues (Sweeney and Klip 1998) as well as the sodium-potassium ATPase pump (Ewart and Klip 1995). Thus, it would be interesting to know whether IGF-I altered either of these, as these would be expected to enhance System A transporter activity.

An additional mechanism, by which IGF-I may elicit its effects on placental transfer, may be through the mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase, which amongst other cell functions, controls cell metabolism and growth through its impacts on transcription and translation and nutrient uptake in many cell types (Regnault, Friedman *et al.* 2005). mTOR has been suggested to act as the nutrient sensor, as it responds to amino acid and glucose sufficiency, energy availability, stress, hypoxia and hormonal stimulation in numerous cell types (Kim, Sarbassov *et al.* 2002). In the presence of high nutrient levels, mTOR activates cell protein synthesis, growth and function, whilst in environments of nutrient deprivation, mTOR is inactive and the cell undergoes catabolic breakdown (Levine, Feng *et al.* 2006). IGFs activate gene expression, protein synthesis (James and Zomerdijk 2004) and glucose transport (Fladeby, Skar *et al.* 2003) via the mTOR signaling pathway in several cell types. Recently, mTOR was identified in human placental tissue (Kim, Sarbassov *et al.* 2002). As IGF abundance is modulated by factors such as nutrition, metabolic state and stress, elevated maternal plasma IGF-I concentration in the current study, may have signaled to the placenta, through activation of mTOR, that the maternal environment is beneficial and to increase substrate supply to the fetus and stimulate fetal growth. Analysis of mTOR and its downstream effectors (such as 4E-binding protein-1 and S6-kinase) in the placenta of IGF treated animals may help elucidate the mechanisms involved.

In addition to acute effects on the fetus and placenta, exogenous maternal IGF-I enhanced MG and AIB uptake by the maternal muscle and spleen. Neither IGF affected maternal weight gain during the treatment. However, maternal IGF-I also increased the absolute and fractional weights of the maternal spleen and kidneys and IGF-II tended to increase the weight of the maternal heart. These alterations,

particularly at the level of nutrient uptake per gram of maternal organ, suggest improved metabolism and thus, functional capacity, which are likely to enhance maternal blood filtration and flow to the uterus and thereby, accelerate conceptus growth. This is consistent with the current findings of increased fetal and placental growth on day 35 with IGF-I and with either IGF treatments on day 40 (Sohlstrom, Fernberg *et al.* 2001).

In contrast to IGF-I, IGF-II treatment did not alter placental transport, fetal or maternal substrate utilization nor fetal or placental growth on day 35 of pregnancy. It is possible that, perhaps analysis on day 35 of gestation was simply too early to see an effect of maternal IGF-II on the fetus and placenta. Indeed, rapid growth of the guinea pig fetus occurs between gestation days 30 and 45 (Kaufmann 1969; Kaufmann and Davidoff 1977) and IGF-II increased fetal and placental growth on day 40 (Sohlstrom, Fernberg *et al.* 2001). In the *Igf2 P0* knock out mouse, effects of this genotype on placental development, transport and fetal growth, were not apparent until after mid gestation (from gestational day 14 and onwards, term = day 20) (Baker, Liu *et al.* 1993; Constancia, Hemberger *et al.* 2002; DeChiara, Efstratiadis *et al.* 1990). It would be interesting to examine placental gene expression or placental invasion of the uterus, to determine what molecular events are taking place in response to maternal IGF-II treatment, which result in enhanced fetal and placental growth on day 40 (Sohlstrom, Fernberg *et al.* 2001). Certainly, maternal IGF-II treatment tended to increase circulating estradiol and progesterone concentrations in the mother, in the current study, suggestive of enhanced placental steroidogenesis.

It is also possible to speculate that the lack of effects of IGF-II on the placenta, fetus and mother may have been related to the extent to which concentrations in the maternal plasma were elevated for the dose administered. In the current study, although the dose of IGF-I and IGF-II administered was the same (1mg/kg/day), the percent increase in circulating IGFs achieved was different due to the greater abundance of IGF-II in normal pregnancy sera. We achieved a 340% increase in circulating IGF-I, while that of IGF-II was increased by just 240%. Furthermore, although the concentration of free IGF to IGFBP ratio in the maternal plasma, and hence bioavailable IGF, was also increased by maternal IGF treatment, the extent to which bioavailable IGF-II was increased compared to vehicle, was almost half that of IGF-I in the IGF-II treated mother. Therefore, the dose of IGF-II administered to the mother may have been too low to exert any acute growth/metabolic effects. To elicit similar effects to those of IGF-I, it may be necessary to elevate circulating and bioavailable IGF-II to a similar level relative to that of IGF-I. Moreover, it is thought that IGF-I is more potent than IGF-II at inducing amino acid uptake by cultured placental trophoblasts (Karl 1995). This also suggests that IGF impacts on substrate uptake occur via

the IGF1R, which IGF-I has greater affinity than IGF-II. IGF-II effects are expected to be distributed across IGF2R, IGF1R and InsR, which it can bind.

Interestingly, maternal IGF-I treatment increased the concentration of IGFBP-3, IGFBP-2 and IGFBP-1 in the maternal circulation. This was surprising as neither IGF treatments altered the maternal circulating total IGFBP binding capacity, in the current study. This difference may reflect reduced sensitivity in measurement of the latter, which was determined indirectly as the interference of the IGFBPs in the IGF radioimmunoassay, as previously utilized by others (Francis, McNeil *et al.* 1989; Francis, Owens *et al.* 1989; Owens, Johnson *et al.* 1990). Nevertheless, elevated concentrations of IGFBPs in the IGF-I treated mother, are expected to prolong the extracellular half-life of IGF-I by preventing its proteolysis (Jones and Clemmons 1995).

Work performed in guinea pigs has shown that the abundance and ratio of IGFs to IGFBP in the maternal circulation are important predictors of placental growth. For instance, Roberts and colleagues have demonstrated that, maternal circulating IGFBP-1 concentrations in mid pregnancy correlate negatively with maternal blood space volume in the exchange region of the placenta (labyrinth) (Roberts, Kind *et al.* 2002). Also a greater availability of IGF-I, reflected by increased IGF-I to IGFBP-3 and IGFBP-1 ratios in these studies, was correlated positively with the development of the placental labyrinth in late pregnancy. Work performed by Prue Standen in the laboratory, has assessed placental structure in the same cohort of guinea pigs as the present study, in response to IGF treatment on day 35 of gestation. Interestingly, her work has suggested that exogenous maternal IGF-I may change the shape of the placenta in mid gestation. IGF-I treatment of the mother reduced the total mid-sagittal cross-sectional area of the placenta by 17% (Standen *et al.*, in preparation), which was largely due to a 22% reduction in the area dedicated for exchange. Despite this, IGF-I did not alter its volume or cellular structure (Standen *et al.*, in preparation). It is postulated that elevated maternal IGF-I may have induced placental trophoblast proliferation instead of differentiation into invasive cells, which is required for expansion of the placental exchange region. Alternatively, a 45% reduction in maternal circulating IGF-II in IGF-I treated mothers, may have indirectly led to altered placental labyrinthine development, as IGF-II has an established role in placental development (Constancia, Hemberger *et al.* 2002; Roberts, Sohlstrom *et al.* 2001b; Sibley, Coan *et al.* 2004) (refer to Chapter 3). This suggests that systemic IGF-I and modulation of its bioavailability by IGFBP-1 and IGFBP-3 or reduced plasma IGF-II abundance within the mother may have influenced placental growth and transporter function.

In conclusion, we have shown that increased maternal circulating IGF-I concentrations from early pregnancy, acutely increases maternal substrate utilization and placental glucose and System A amino acid transfer to the fetus, enhancing fetal and placental growth. While IGF-II does not induce immediate effects on the fetus, placenta or mother, in the current study on day 35 of gestation, this treatment has been reported to have anabolic effects a few days later (Sohlstrom, Fernberg *et al.* 2001). Further studies are clearly required to determine whether the effects of IGF-I in the present study, as well as IGF-II in a previous study (Sohlstrom, Fernberg *et al.* 2001), are sustained until term, after cessation of treatment. These promise to advance our understanding of the endocrine regulation of fetal growth by the IGFs through the placenta, acting as a nutrient sensor, and the mother supporting the process of pregnancy. This may ultimately help understand the pathophysiology of pregnancies complicated by growth restriction.



*Chapter 5 : Sustained effects of  
maternal IGF treatment on maternal  
and fetal body composition and  
placental development*

## 5.1 Introduction

The placenta is a multifunctional organ that forms the interface between the fetal and maternal circulations. It is essential for fetal growth as it supplies the developing fetus with oxygen and nutrients, transporting them from the mother into the umbilical circulation. Abnormalities in placental structural development can impair placental function, reducing substrate supply to the fetus and may result in intrauterine growth restriction (IUGR) (Khong, De Wolf *et al.* 1986). It is estimated that placental dysfunction accounts for 70-80% of growth restricted newborns (Regnault, de Vrijer *et al.* 2002a), currently affecting 6% of pregnancies in developed countries (Laws and Sullivan 2004) and up to 40% in developing countries (Albertsson-Wikland, Wennergren *et al.* 1993). IUGR is associated with perinatal morbidity and mortality and increases the risk of poor health in childhood and adult life (Barker 1998; Barker 2004a; Barker 2004c). In addition, impaired placental trophoblast invasion of the maternal uterine vasculature and/or poor placental function are implicated in other major pregnancy complications, miscarriage (Khong, Liddell *et al.* 1987), preeclampsia (Khong 2000; Khong, De Wolf *et al.* 1986), placental abruption (Dommissse and Tiltman 1992), preterm labour (Kim, Bujold *et al.* 2003; Kim, Chaiworapongsa *et al.* 2002) and stillbirth (Khong, De Wolf *et al.* 1986; Robertson, Brosens *et al.* 1967). Therefore, it is imperative that we understand the factors essential for regulating placental functional development to identify causes of such diseases and as a basis for the development of therapeutics.

The insulin-like growth factors (IGF)-I and IGF-II, have been implicated in placental structural and functional development. *Igf2* over-expression in mice causes placental and fetal overgrowth (Ferguson-Smith, Cattnach *et al.* 1991), while *Igf2* gene deletion reduces placental weight by 17% on day 13.5 and 25% on day 16.5 of gestation, with a fetal weight reduction of 40% from day 16.5 (term=19 days) (Baker, Liu *et al.* 1993; DeChiara, Efstratiadis *et al.* 1990). In addition, placental amino acid transporter expression is altered by *Igf2* deficiency in mice (Matthews, Beveridge *et al.* 1999). Ablation of the placental specific *Igf2* promoter (P0) in mice, reduces placental weight and adversely affects placental structural differentiation and transport capacity with reduced fetal weight evident two days later (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004). The latter reduction in fetal weight was comparable to that induced by global *Igf2* gene ablation, suggesting that the effects of *Igf2* deficiency on fetal growth are mediated by actions on the placenta in mice.

In contrast, *Igf1* gene ablation in mice does not alter placental weight but reduces fetal weight, indicating that IGF-I is important in the fetus (Baker, Hardy *et al.* 1996; Liu, Baker *et al.* 1993). IGF-I may modulate

placental nutrient capacity since IGF-I administration to pregnant rats, or increased endogenous expression in pregnant mice, increases the weight of the fetus but not that of the placenta (Gluckman and Harding 1992). IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (Bloxam, Bax *et al.* 1994; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998) and promotes placental nutrient uptake and metabolism when infused into fetal sheep (Bloomfield, Zijl *et al.* 2002; Harding, Liu *et al.* 1994; Liechty, Boyle *et al.* 1996). Moreover, exposure to IGF-I inhibits release of vasoconstrictors, such as thromboxane B<sub>2</sub> and prostaglandin F<sub>2</sub>  $\alpha$ , in human term placental explants (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995), which may increase placental blood flow and delivery of nutrients for the growth of the fetus.

The placenta is exposed to IGFs from multiple sources, including those produced locally and those circulating within the fetus and mother. Maternally derived IGFs may have a major influence on placental development, particularly in women and in guinea pigs where circulating IGFs are substantial (Gargosky, Moyses *et al.* 1990; Sohlstrom, Katsman *et al.* 1998b). Indeed, the IGF axis in guinea pigs is very similar to that of humans (Keightley and Fuller 1996), whereas rats and mice do not have circulating IGF-II postnatally. The placenta in guinea pigs is more similar to the human placenta than that of other non-primate species being haemomonochorial, although it is labyrinthine rather than villous in structure. The guinea pig placenta is comprised of a labyrinth, which contains both fetal capillaries and maternal blood sinuses and provides the means for exchange between the two circulations and an interlobium which is comprised of syncytiotrophoblast and maternal blood sinuses, and is the site where much of the metabolic activity of the placenta is thought to occur (Kaufmann 1969; Kaufmann and Davidoff 1977). In the human placenta, exchange and endocrine functions are performed in the placental villi (Gude, Roberts *et al.* 2004). In addition, placental trophoblast cells in guinea pigs are highly invasive and, like those in humans, engage in interstitial and endovascular invasion of the decidua. They remodel the uterine spiral arterioles to permit the large increase in blood flow to the placenta (Moll, Espach *et al.* 1983; Nanaev, Chwalisz *et al.* 1995a) that is essential for placental growth and subsequent function and therefore fetal growth.

In the guinea pig, major structural determinants of placental function are strongly predicted by maternal IGF-II concentration in mid pregnancy and by maternal IGF-I in late pregnancy (Roberts, Kind *et al.* 2002; Roberts, Sohlstrom *et al.* 2001b). Furthermore, in this species, food restriction reduces maternal plasma IGF concentrations (Sohlstrom, Katsman *et al.* 1998b) which correlate with delayed structural and functional maturation of the placenta and with reduced fetal growth (Roberts, Sohlstrom *et al.*

2001a). The structural defects in the placenta in food restricted guinea pigs are similar to those seen in placentas from women with preeclampsia (Roberts, Sohlstrom *et al.* 2001a). In addition, reduced maternal plasma IGF-I in pregnant women is associated with placental dysfunction and small for gestational age (Larsen, Main *et al.* 1996; Stefanidis, Solomou *et al.* 1998) or growth restricted infants (Holmes, Montemagno *et al.* 1997).

Consistent with these adaptive changes in maternal IGFs regulating placental development, maternal supplementation with IGF-I or IGF-II in early to mid pregnancy in the guinea pig increases placental and fetal weights by mid gestation (Sohlstrom, Fernberg *et al.* 2001) (Chapter 4). We therefore suggest that the increased maternal production of both IGFs in early pregnancy is an important adaptation to pregnancy, which promotes placental functional development and consequently fetal growth. Whether anabolic effects of an increased abundance of maternal IGFs in early pregnancy on the placenta would persist into late gestation and affect the fetus is currently unknown. Furthermore, in contrast to IGF-I, which binds almost exclusively to the type 1 IGF receptor (IGF1R) and mediates its effects on fetal growth (Baker, Liu *et al.* 1993), IGF-II cross-reacts with both IGF receptors (IGF1R and IGF2R) and the insulin receptor (InsR), hindering the identification of the receptor potentiating IGF-II actions. Therefore, the aims of this study were to 1) determine the effects of maternal IGF-I and IGF-II supplementation in early to mid pregnancy on placental development and fetal growth and viability near term and 2) determine the role of IGF2R in mediating IGF-II influences on fetal and placental growth. The latter aim was undertaken using, [Leu<sup>27</sup>]-IGF-II (Leu<sup>27</sup>-IGF-II), an IGF-II analogue which selectively interacts with IGF2R (Sakano, Enjoh *et al.* 1991).

## 5.2 Results

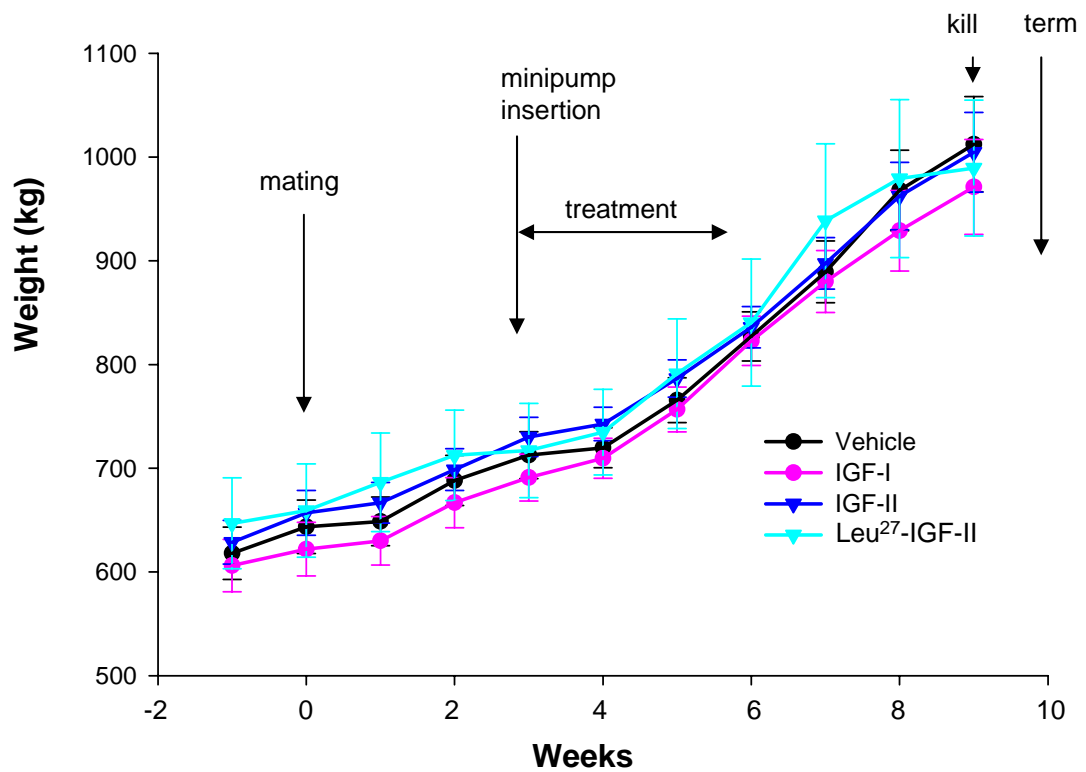
### *5.2.1.1 Sustained effects of exogenous IGF treatment to the mother on maternal body composition and weight gain during pregnancy*

Pregnant guinea pigs were infused with IGF-I, IGF-II, Leu<sup>27</sup>-IGF-II (each 1mg/kg/day) or vehicle (0.1M acetic acid) subcutaneously from days 20 to 38 of pregnancy and killed on day 62 (term = 69 days). This treatment elevated maternal circulating IGF-I and IGF-II concentrations by 2.4 and 3.4 fold, respectively (Chapter 4, p 109).

To determine whether exogenous IGFs affected the mother, maternal weight gain and body composition analyses were performed. Both exogenous maternal IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II did not alter maternal weight gain during or following IGF treatment (Figure 5.1), nor total body and lean body mass near term (Table 5.1). IGF-I reduced maternal interscapular fat depot weight (-25%,  $P = 0.028$ ) and the fractional weights of the perirenal (-32%,  $P = 0.05$ ), retroperitoneal (-33%,  $P = 0.037$ ) and interscapular fat (-28%,  $P = 0.01$ , Table 5.1). IGF-I reduced the absolute and fractional weights of the combined adipose depot weights in the mother by -30%, ( $P = 0.016$  and  $P = 0.007$ , respectively). Leu<sup>27</sup>-IGF-II reduced the absolute and fractional weights of the maternal retroperitoneal fat (-34%,  $P = 0.04$  and -32%,  $P = 0.02$ , respectively) and the absolute weight of the maternal interscapular fat (-18%,  $P = 0.05$ ). IGF-II treatment tended to increase the absolute weight of the spleen (+31%,  $P = 0.056$ ) but did not affect any other tissue or organ weight in absolute or relative to body weight terms (Table 5.1).

### *5.2.1.2 Sustained effects of exogenous maternal IGF treatment on maternal circulating hormone concentrations*

To determine whether treatment of the mother during early to mid pregnancy with IGFs altered maternal circulating estradiol (Figure 5.2a) and progesterone (Figure 5.2b) near term, specific radioimmunoassays were performed. Treating the mother during early to mid pregnancy with IGF-I doubled circulating maternal estradiol concentrations in late pregnancy although did not reach significance ( $P = 0.078$ ). IGF-I treatment did not alter late pregnancy circulating progesterone concentration. Both exogenous maternal IGF-II and Leu<sup>27</sup>-IGF-II during early to mid pregnancy did not alter circulating estradiol or progesterone concentrations in the mother in late pregnancy (Figure 5.2).



**Figure 5.1 Sustained effect of maternal IGF treatment in early to mid pregnancy on maternal weight gain during pregnancy.** Female guinea pigs were weighed three times weekly during the study to determine an average weekly weight, from one week before mating and during pregnancy up until kill. Mini osmotic pumps were inserted on day 20 of pregnancy and delivered vehicle ( $n = 7$ ), IGF-I ( $n = 7$ ), IGF-II ( $n = 9$ ) or Leu<sup>27</sup>-IGF-II ( $n = 4$ ) for 18 days. Term, which is ~67-70 days of pregnancy, is denoted on the graph. Values are expressed as means  $\pm$  SEM. Repeated measures ANOVA was performed and found no significant effect of treatment.

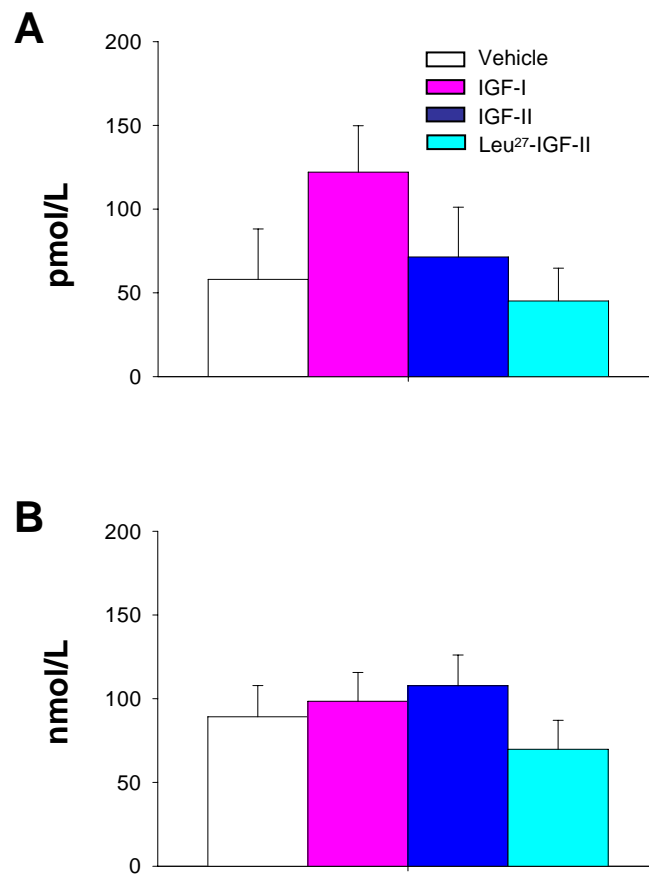
**Table 5.1 Sustained effect of maternal IGF treatment in early to mid pregnancy on maternal body composition**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number of dams	7	7	9	4
Weight at d62	978 ± 23	1012 ± 34	971 ± 36	982 ± 49
Net body mass**	736 ± 21	761 ± 62	725 ± 19	691 ± 36
Lean body mass†	711 ± 19	744 ± 62	702 ± 18	671 ± 48
Adrenals (g)	55 ± 0.06	56 ± 0.06	54 ± 0.06	64 ± 0.06
(% Body weight)†	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.09 ± 0.00
Kidneys (g)	4.66 ± 0.2	4.85 ± 0.2	4.94 ± 0.2	4.74 ± 0.2
(% Body weight)†	6.3 ± 0.03	6.5 ± 0.03	6.8 ± 0.03	6.9 ± 0.04
Spleen (g)	1.29 ± 0.1 <sup>a</sup>	1.32 ± 0.1 <sup>a</sup>	1.69 ± 0.1 <sup>c</sup>	1.25 ± 0.2 <sup>a</sup>
(% Body weight)†	0.17 ± 0.01	0.17 ± 0.02	0.23 ± 0.01	0.18 ± 0.02
Pancreas (g)	1.89 ± 0.3	2.12 ± 0.2	2.13 ± 0.2	1.92 ± 0.3
(% Body weight)†	0.26 ± 0.03	0.28 ± 0.03	0.29 ± 0.03	0.28 ± 0.04
Liver (g)	26.6 ± 1.3	26.5 ± 1.2	26.8 ± 1.1	27.1 ± 1.6
(% Body weight)†	3.9 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	3.9 ± 0.2
Lung (g)	4.35 ± 0.4	3.84 ± 0.4	4.40 ± 0.3	5.14 ± 0.5
(% Body weight)†	0.58 ± 0.06	0.51 ± 0.06	0.60 ± 0.05	0.77 ± 0.08
Brain (g)	3.95 ± 0.1	4.12 ± 0.1	4.12 ± 0.1	3.96 ± 0.1
(% Body weight)†	0.53 ± 0.02	0.56 ± 0.03	0.56 ± 0.02	0.58 ± 0.03
Heart (g)	2.16 ± 0.2	2.06 ± 0.2	2.51 ± 0.2	2.36 ± 0.3
(% Body weight)†	0.29 ± 0.03	0.27 ± 0.03	0.34 ± 0.03	0.34 ± 0.04
Uterus* (g)	231 ± 38	250 ± 35	221 ± 33	291 ± 47
(% Body weight)†	0.32 ± 0.06	0.36 ± 0.05	0.30 ± 0.05	0.42 ± 0.08
Ovaries (g)	0.13 ± 0.01	0.16 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
(% Body weight)†	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Stomach (g)	7.44 ± 0.7	5.80 ± 0.7	6.33 ± 0.6	6.58 ± 0.9
(% Body weight)†	0.68 ± 0.3	0.56 ± 0.3	0.87 ± 0.3	0.95 ± 0.3
S. intestine (g)	17.9 ± 1.3	17.6 ± 1.2	14.6 ± 1.1	17.1 ± 1.6
(% Body weight)†	2.4 ± 0.02	2.3 ± 0.01	2.0 ± 0.02	2.5 ± 0.02

L. intestine (g)	8.51 ± 1.3	8.25 ± 1.2	10.28 ± 1.1	6.93 ± 1.6
(% Body weight) <sup>‡</sup>	1.2 ± 0.2	1.1 ± 0.2	1.4 ± 0.2	1.0 ± 0.2
Caecum (g)	11.2 ± 0.9	10.9 ± 0.8	10.0 ± 0.8	11.4 ± 1.1
(% Body weight) <sup>‡</sup>	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.7 ± 0.2
Total GIT (g)	45.0 ± 3.1	42.6 ± 2.3	41.2 ± 2.2	42.0 ± 2.5
(% Body weight) <sup>‡</sup>	6.1 ± 0.4	5.7 ± 0.3	5.7 ± 0.3	6.1 ± 0.4
M. soleus (g)	0.17 ± 0.03	0.18 ± 0.3	0.17 ± 0.03	0.19 ± 0.04
(% Body weight) <sup>‡</sup>	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
M. biceps (g)	0.44 ± 0.03	0.48 ± 0.03	0.44 ± 0.03	0.46 ± 0.04
(% Body weight) <sup>‡</sup>	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.07 ± 0.00
M. triceps (g)	2.13 ± 0.10	2.26 ± 0.98	2.33 ± 0.09	2.24 ± 0.13
(% Body weight) <sup>‡</sup>	0.29 ± 0.01	0.30 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
M. gastroc (g)	1.94 ± 0.10	1.99 ± 0.09	1.97 ± 0.08	1.88 ± 0.12
(% Body weight) <sup>‡</sup>	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.27 ± 0.01
Perirenal fat (g)	5.27 ± 0.8 <sup>a</sup>	3.50 ± 0.5 <sup>b</sup>	4.72 ± 0.4 <sup>ab</sup>	5.17 ± 0.94 <sup>a</sup>
(% Body weight) <sup>‡</sup>	0.71 ± 0.1 <sup>a</sup>	0.48 ± 0.08 <sup>b</sup>	0.66 ± 0.05 <sup>ab</sup>	0.72 ± 0.12 <sup>a</sup>
Retro fat (g)	8.96 ± 0.9 <sup>a</sup>	6.27 ± 0.8 <sup>b</sup>	8.47 ± 0.6 <sup>ab</sup>	5.87 ± 1.1 <sup>b</sup>
(% Body weight) <sup>‡</sup>	1.2 ± 0.1 <sup>a</sup>	0.85 ± 0.1 <sup>b</sup>	1.2 ± 0.06 <sup>a</sup>	0.82 ± 0.12 <sup>b</sup>
Inter fat (g)	10.85 ± 0.9 <sup>a</sup>	8.11 ± 0.6 <sup>b</sup>	9.95 ± 0.4 <sup>ab</sup>	8.94 ± 1.1 <sup>b</sup>
(% Body weight) <sup>‡</sup>	1.5 ± 0.1 <sup>a</sup>	1.1 ± 0.08 <sup>b</sup>	1.4 ± 0.06 <sup>a</sup>	1.3 ± 0.13 <sup>ab</sup>
Total fat (g)	25.2 ± 2.2 <sup>a</sup>	17.9 ± 2.1 <sup>b</sup>	23.4 ± 1.9 <sup>ab</sup>	20.0 ± 2.7 <sup>ab</sup>
(% Body weight) <sup>‡</sup>	3.4 ± 0.27 <sup>a</sup>	2.4 ± 0.25 <sup>b</sup>	3.2 ± 0.23 <sup>a</sup>	2.8 ± 0.33 <sup>ab</sup>
Skin (g)	112 ± 7.8	111 ± 6.6	115 ± 6.2	102 ± 8.7
(% Body weight) <sup>‡</sup>	15 ± 1.1	15 ± 0.9	16 ± 0.9	15 ± 1.3
Carcass (g)	418 ± 16	383 ± 15	395 ± 14	371 ± 19
(% Body weight) <sup>‡</sup>	56.6 ± 2.0	51.1 ± 1.9	54.0 ± 1.7	53.8 ± 2.5

Data expressed as means ± SEM. <sup>†</sup>Uterus and contents. <sup>\*\*</sup>Net body mass is weight at post-mortem minus the uterus and contents. <sup>†</sup>Lean body mass is net body mass minus total fat. <sup>‡</sup>Tissue weight calculated as a percentage of net body mass. GIT, gastrointestinal tract; Inter fat, interscapular fat; L. intestine, large intestine; M.gastroc, M.gastrocnemius; Retro fat, retroperitoneal fat; S. intestine, small intestine. Different superscripts denote difference between groups, a vs b, P < 0.05 (significant), a vs c P = 0.056 (not significant), as determined by Univariate ANOVA with Bonferoni Post Hoc Tests.





**Figure 5.2** Sustained effect of exogenous maternal IGFs in early to mid pregnancy on circulating hormone concentrations in the mother. Estradiol (a) and progesterone (b) plasma concentrations were determined. Data are from vehicle (n = 7), IGF-I (n = 7), IGF-II (n = 9) and Leu<sup>27</sup>-IGF-II (n = 4) treated mothers and values are expressed as means  $\pm$  SEM. Data was analysed by a General Linear Univariate ANOVA with Bonferoni Post Hoc Tests.

### 5.2.1.3 Sustained effects of maternal IGF treatment on the development of the placental exchange region (labyrinth)

IGF treatment in early to mid pregnancy did not alter placental weight in late gestation, compared with vehicle (Table 5.2). Placentae from IGF-II treated mothers were 18-19% heavier than those from mothers treated with IGF-I and Leu<sup>27</sup>-IGF-II (P = 0.034 and P = 0.05, respectively).

To elucidate whether exogenous IGF affected placental structure, the two regions of the guinea pig placenta, the labyrinth and interlobium, were identified by Masson's Trichrome staining. Exogenous IGF-II increased placental labyrinthine cross-sectional area by 28% (P = 0.005) but not that of the interlobium (Figure 5.3a-d, Table 5.2). Leu<sup>27</sup>-IGF-II treatment tended to reduce the cross-sectional area of the interlobium (P = 0.07), while neither IGF-II nor Leu<sup>27</sup>-IGF-II treatment altered the total placental cross-sectional area (Table 5.2). The ratio of labyrinth to interlobium was increased by IGF-II (+37%, P = 0.054) and Leu<sup>27</sup>-IGF-II (+39%, P = 0.017). IGF-II and Leu<sup>27</sup>-IGF-II treatments both increased the proportion of the placenta comprised of labyrinth (both +9%, P < 0.003) and reduced that composed of the interlobium (both -24%, P < 0.003) (Table 5.2). IGF-II also increased the volume of placental labyrinth (+28%, P = 0.027) but did not alter that of the interlobium, while Leu<sup>27</sup>-IGF-II treatment reduced the volume of the interlobium (-33%, P = 0.01, Table 5.2). Maternal IGF-I treatment did not alter any placental parameter (Table 5.2).

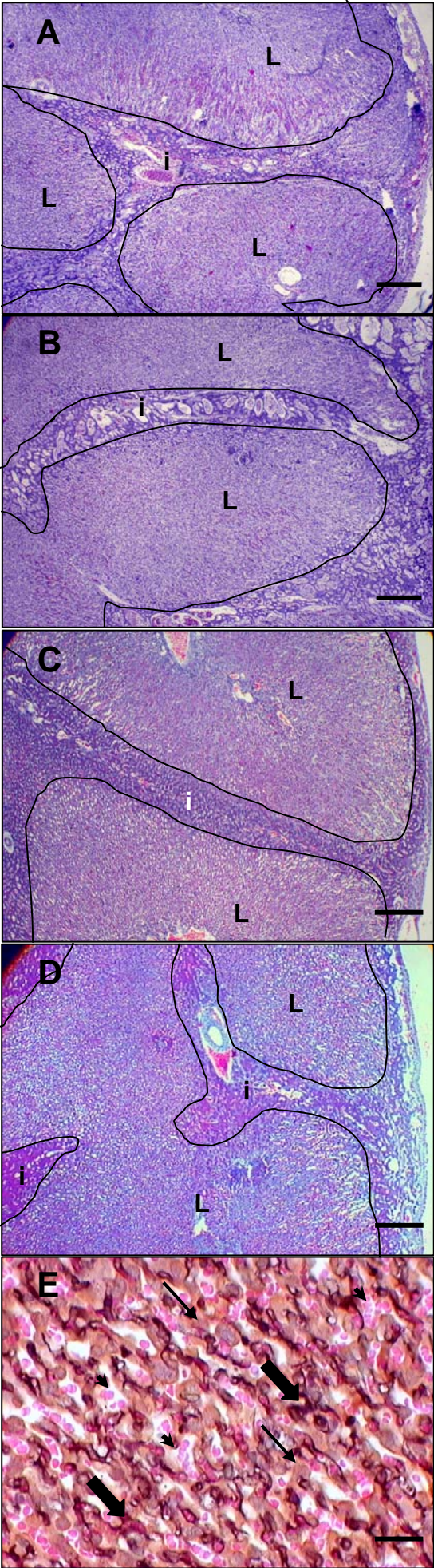
To examine placental labyrinthine development in response to earlier maternal IGFs, structural components of the placental labyrinth, fetal trophoblast, maternal blood spaces and fetal capillaries, were identified using double-label immunohistochemistry and haematoxylin and eosin counter-staining and structural correlates of placental function were quantified using video image analysis (Figure 5.3e). Maternal Leu<sup>27</sup>-IGF-II treatment increased the proportion of the labyrinth composed of trophoblast and maternal blood spaces (+22%, P = 0.014 and +40%, P = 0.0003, respectively), while reducing that of fetal capillaries (-60%, P < 0.0001), however neither IGF-I nor IGF-II treatment had an effect (Figure 5.4a). Both IGF-II and Leu<sup>27</sup>-IGF-II treatments increased the volume of trophoblast (+29%, P = 0.015 and +27%, P = 0.014, respectively) and that of maternal blood spaces (+46%, P = 0.035 and +40%, P = 0.003, respectively) within the placental labyrinth (Figure 5.4b).

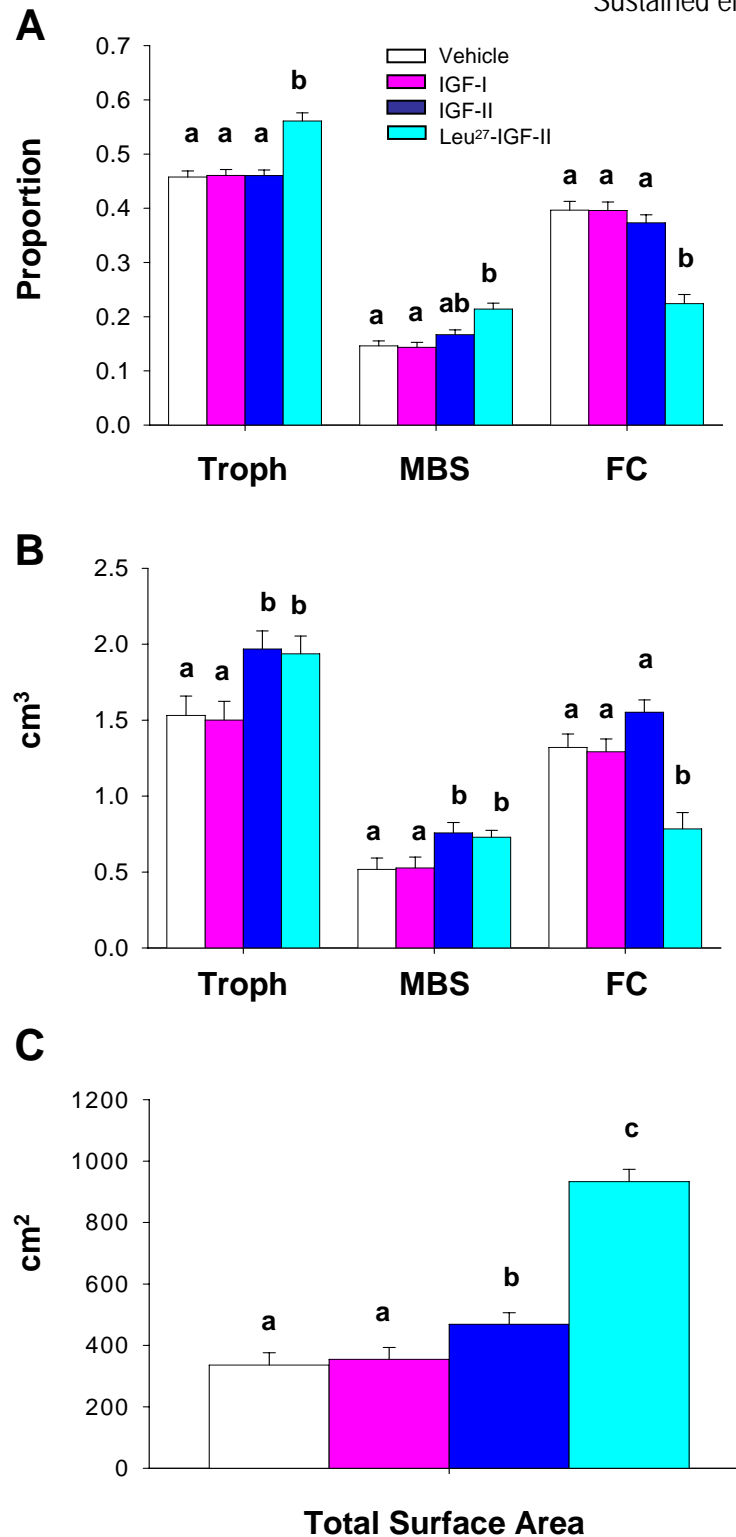
**Table 5.2 Sustained effect of maternal IGF treatment in early to mid pregnancy on placental structure**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number of dams	7	7	9	4
Placental weight (g)	4.63 ± 0.29 <sup>ab</sup>	4.11 ± 0.24 <sup>a</sup>	4.84 ± 0.22 <sup>b</sup>	4.07 ± 0.33 <sup>a</sup>
Cross-sectional area labyrinth (mm <sup>2</sup> )	98.9 ± 3.8 <sup>a</sup>	112.3 ± 8.9 <sup>a</sup>	126.6 ± 8.3 <sup>b</sup>	108.8 ± 8.4 <sup>ab</sup>
Cross-sectional area interlobium (mm <sup>2</sup> )	35.6 ± 2.8	32.6 ± 4.3	30.4 ± 2.6	25.8 ± 2.9
Total cross-sectional area (mm <sup>2</sup> )	136 ± 14	145 ± 11	157 ± 12	135 ± 10
Labyrinth : Interlobium	3.10 ± 0.43 <sup>a</sup>	3.89 ± 0.44 <sup>a</sup>	4.23 ± 0.35 <sup>c</sup>	4.31 ± 0.33 <sup>b</sup>
Proportion labyrinth (%)	73.6 ± 1.2 <sup>a</sup>	77.6 ± 1.1 <sup>ab</sup>	80.5 ± 1.1 <sup>b</sup>	80.6 ± 1.4 <sup>b</sup>
Proportion interlobium (%)	26.4 ± 1.2 <sup>a</sup>	22.4 ± 1.1 <sup>ab</sup>	19.5 ± 1.1 <sup>b</sup>	19.4 ± 1.5 <sup>b</sup>
Volume labyrinth (cm <sup>3</sup> )	3.34 ± 0.25 <sup>a</sup>	3.26 ± 0.23 <sup>a</sup>	4.26 ± 0.23 <sup>b</sup>	3.44 ± 0.21
Volume interlobium (cm <sup>3</sup> )	1.21 ± 0.09 <sup>a</sup>	0.95 ± 0.09 <sup>ab</sup>	1.03 ± 0.08 <sup>ab</sup>	0.81 ± 0.1 <sup>b</sup>

Data are expressed as mean ± SEM with 1-3 placentae per dam randomly selected for histological analysis. Different superscripts denote differences between groups, a vs b,  $P < 0.039$  (significant) and a vs c,  $P = 0.054$  (not significant). Statistical analyses were performing using Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject and the fetus as the repeated measure.

**Figure 5.3 Photomicrographs demonstrating sustained effect of exogenous maternal IGF treatment in early to mid pregnancy on placental structure.** Representative mid-sagittal sections of near term placentae stained with Masson's Trichrome to distinguish labyrinth and interlobium layers from mothers that had been treated with vehicle (**a**), IGF-I (**b**) IGF-II (**c**) or Leu<sup>27</sup>-IGF-II (**d**) during early to mid pregnancy. L, labyrinth; i, interlobium. Scale bars = 400  $\mu$ m. Representative mid-sagittal section of near term placenta double-immunolabelled with haematoxylin and eosin counter-staining shown (**d**) to reveal structural components of the placental labyrinth, including fetal trophoblast (*thin arrows*), maternal blood spaces (*asterisks*) and fetal capillaries (*broad arrows*). Scale bar = 40  $\mu$ m.





**Figure 5.4** Sustained effect of exogenous maternal IGFs in early to mid pregnancy on structural correlates of placental exchange function. Proportions (a) and volumes (b) of fetal trophoblast, maternal blood spaces and fetal capillaries in the placental labyrinth (exchange region), as well as the total surface area of syncytiotrophoblast for exchange (c). Data are from  $n = 1-3$  placentae from each mother ( $n = 4-9$  dams per treatment). Values are expressed as means  $\pm$  SEM. Different superscripts denote statistically significant differences,  $P < 0.05$ , as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject and the fetus as a repeated measure.

Treatment of the mother with Leu<sup>27</sup>-IGF-II also reduced the volume of labyrinthine fetal capillaries (-40%,  $P = 0.017$ , Figure 5.4b). The total surface area of trophoblast functioning in exchange was also increased by IGF-II and Leu<sup>27</sup>-IGF-II treatments (+39%,  $P = 0.037$  and +277%,  $P < 0.0001$ , Figure 5.4c). Leu<sup>27</sup>-IGF-II treatment reduced syncytiotrophoblast barrier thickness, compared to vehicle and both IGF-I and IGF-II treatments (vehicle:  $4.7 \pm 0.2 \mu\text{m}$ , IGF-I:  $4.8 \pm 0.2 \mu\text{m}$ ; IGF-II:  $4.4 \pm 0.2 \mu\text{m}$  and Leu<sup>27</sup>-IGF-II:  $2.1 \pm 0.3 \mu\text{m}$ ,  $P < 0.001$ ). Maternal IGF-I treatment did not affect any placental labyrinthine structural parameter measured.

#### *5.2.1.4 Sustained effects of exogenous maternal IGFs on litter size and fetal survival*

Maternal IGF treatment did not affect total litter size (Table 5.3). However, IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treatment reduced the number of resorptions (-77%,  $P = 0.009$ , -60%,  $P = 0.01$ , -100%,  $P = 0.033$ , respectively) and increased the number of viable fetuses (+25%,  $P = 0.011$ , +23%,  $P = 0.019$ , +27%,  $P = 0.05$ , respectively) near term (Table 5.3). There was no effect of treatment on the ratio of females to males (Table 5.3).

#### *5.2.1.5 Sustained effects of exogenous maternal IGFs on fetal growth*

Maternal IGF-I and IGF-II treatment in early to mid pregnancy increased fetal weight near term by 17% ( $P = 0.002$ ) and 11% ( $P = 0.042$ ), respectively (Table 5.4). Maternal IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treatments, significantly skewed the fetal weight distribution to the right (all  $P < 0.0005$ , Figure 5.5a). The percentage of fetuses heavier than 81 grams was 5% in controls, 37% in IGF-I, 19% in IGF-II and 9% in Leu<sup>27</sup>-IGF-II treated animals (Figure 5.5a). IGF-I treatment increased fetal crown-rump length by 9% ( $P = 0.014$ ), as well as abdominal circumference by 10% ( $P = 0.05$ ). IGF-I increased the fetal weight to placental weight ratio by 29% compared with vehicle and IGF-II treatment groups (vehicle:  $14.82 \pm 0.86$ ; IGF-I:  $19.14 \pm 0.73$ ; IGF-II:  $16.18 \pm 0.65$ ; Leu<sup>27</sup>-IGF-II:  $17.72 \pm 0.92$ ,  $P < 0.001$  and  $P = 0.007$ ). Fetal weight correlated positively with placental weight across all treatments ( $r = 0.27$ ,  $P = 0.026$ ) and within IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treatment groups ( $r = 0.44$ ,  $P = 0.042$ ;  $r = 0.51$ ,  $P = 0.005$  and  $r = 0.84$ ,  $P = 0.0003$ , respectively) but not in vehicle treated dams alone (Figure 5.5b).

**Table 5.3 Sustained effect of maternal IGF treatment in early to mid pregnancy on litter composition**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Dams	7	7	9	4
Fetuses	19	22	30	13
F / M*	9 / 10	12 / 10	14 / 16	9 / 4
Total Litter	3.42 ± 0.1	3.36 ± 0.1	3.67 ± 0.1	3.46 ± 0.2
Number viable	2.73 ± 0.2 <sup>a</sup>	3.42 ± 0.2 <sup>b</sup>	3.37 ± 0.1 <sup>b</sup>	3.46 ± 0.2 <sup>b</sup>
Number resorbing	0.68 ± 0.1 <sup>a</sup>	0.09 ± 0.1 <sup>b</sup>	0.27 ± 0.1 <sup>b</sup>	0.00 ± 0.00 <sup>b</sup>

Data are expressed as mean ± SEM. \*F = females, M = males. Different superscripts denote significant differences between groups, a vs b P < 0.05. Statistical analyses were performing using Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject and the fetus as the repeated measure.



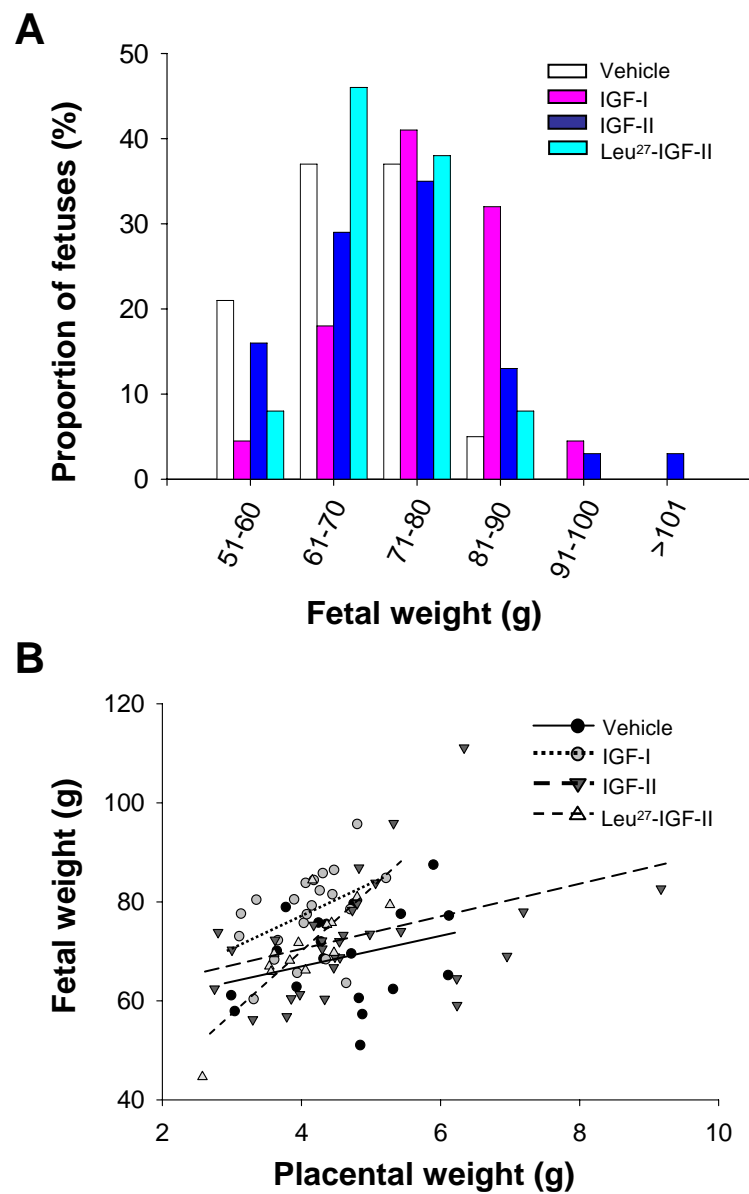


Figure 5.5 Sustained effect of maternal IGF treatment in early to mid pregnancy on fetal weight distribution (a) and the association of fetal and placental weights (b). Regression curves are displayed for vehicle, IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treated animals. Each fetus per treatment is represented (n = 4-9 mothers per treatment). Refer to text for r and P values.

Overall, fetal weight correlated positively with both the mid-sagittal cross-sectional area and the estimated total volume of the placental labyrinth ( $r = 0.638$ ,  $P = 0.001$  and  $r = 0.61$ ,  $P < 0.0001$ , respectively), as well as with the volumes of trophoblast and fetal capillaries in the placental labyrinth (both  $r = 0.51$ ,  $P = 0.001$ ).

#### *5.2.1.6 Sustained effects of exogenous maternal IGFs on fetal body composition*

Maternal IGF-I treatment increased fetal carcass weight (+19%,  $P = 0.002$ ), increased the combined weights of fetal kidneys (+20%,  $P = 0.028$ ), caecum (+24%,  $P = 0.027$ ), total gastrointestinal tract (+13.5%,  $P = 0.049$ ) and the combined fetal fat depots (+16%,  $P = 0.028$ ) (Table 5.4). Conversely, IGF-I reduced the fractional weights of the fetal spleen (-24%,  $P = 0.001$ ), liver (-12.5%,  $P = 0.002$ ) and brain (-18.5%,  $P = 0.004$ ) (Table 5.4). Both IGF-I and IGF-II increased the weights of the fetal retroperitoneal fat (+24%,  $P = 0.004$ ; +18%,  $P = 0.031$ , respectively), combined muscle mass (+22%,  $P = 0.008$ ; +19%,  $P = 0.024$ , respectively) and the triceps absolute (+29%,  $P = 0.001$ ; +24%  $P = 0.01$ , respectively) and relative weights (both +16%,  $P < 0.03$ , Table 5.4). Compared to vehicle, Leu<sup>27</sup>-IGF-II increased the total weight of the gastrointestinal tract (+15%,  $P = 0.048$ ) and reduced the fractional weight of the fetal liver compared with the vehicle treatment group (-16%,  $P = 0.004$ ). Leu<sup>27</sup>-IGF-II increased the absolute and fractional weights of the fetal perirenal (+20%,  $P = 0.05$ ; +17%,  $P = 0.033$ , respectively), interscapular (+23%,  $P = 0.025$ ; +20%,  $P = 0.05$ , respectively), retroperitoneal (+21%,  $P = 0.05$ ; +22%,  $P = 0.05$ , respectively) and combined fat depots (+25.5%,  $P = 0.01$ ; +19%,  $P = 0.033$ , respectively). There was no effect of treatment on the fetal liver weight to brain weight ratio (estimated marginal mean  $\pm$  SEM; vehicle:  $1.51 \pm 0.1$ ; IGF-I:  $1.52 \pm 0.7$ ; IGF-II:  $1.53 \pm 0.08$ ; Leu<sup>27</sup>-IGF-II:  $1.35 \pm 0.9$ ). Body composition of male and female fetuses was similar and was similarly affected by maternal IGF and Leu<sup>27</sup>-IGF-II treatment (data not shown).

**Table 5.4 Sustained effect of maternal IGF treatment in early to mid pregnancy on fetal weight and body composition**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Fetal weight (g)	66.62 ± 2.40 <sup>a</sup>	77.75 ± 1.96 <sup>b</sup>	74.03 ± 1.69 <sup>b</sup>	70.66 ± 2.77 <sup>ab</sup>
Crown-rump length (cm)	14.00 ± 0.34 <sup>a</sup>	15.28 ± 0.28 <sup>b</sup>	14.77 ± 0.24 <sup>ab</sup>	13.87 ± 0.43 <sup>ab</sup>
Abdominal circ. (cm)	8.82 ± 0.28 <sup>a</sup>	9.69 ± 0.23 <sup>b</sup>	9.01 ± 0.20 <sup>ab</sup>	9.23 ± 0.28 <sup>ab</sup>
Head width (cm)	6.81 ± 0.46	7.07 ± 0.39	7.20 ± 0.37	7.20 ± 0.51
Adrenals (g)	0.055 ± 0.01	0.028 ± 0.01	0.036 ± 0.01	0.026 ± 0.01
(% Body weight)	0.08 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
Kidneys (g)	0.59 ± 0.04 <sup>a</sup>	0.71 ± 0.03 <sup>b</sup>	0.67 ± 0.03 <sup>ab</sup>	0.56 ± 0.04 <sup>a</sup>
(% Body weight)	0.89 ± 0.04	0.92 ± 0.03	0.91 ± 0.03	0.79 ± 0.04
Spleen (g)	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.91 ± 0.04
(% Body weight)	0.17 ± 0.01 <sup>a</sup>	0.13 ± 0.01 <sup>b</sup>	0.15 ± 0.01 <sup>ab</sup>	0.13 ± 0.06 <sup>ab</sup>
Pancreas (g)	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
(% Body weight)	0.15 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.14 ± 0.02
Liver (g)	3.71 ± 0.18	3.77 ± 0.14	3.84 ± 0.13	3.34 ± 0.20
(% Body weight)	5.6 ± 0.2 <sup>a</sup>	4.9 ± 0.1 <sup>b</sup>	5.2 ± 0.1 <sup>ab</sup>	4.7 ± 0.02 <sup>b</sup>
Lungs (g)	1.33 ± 0.05	1.42 ± 0.04	1.31 ± 0.04	1.36 ± 0.06
(% Body weight)	1.9 ± 0.1	1.9 ± 0.04	1.9 ± 0.04	1.9 ± 0.1
Brain (g)	2.49 ± 0.07	2.51 ± 0.06	2.52 ± 0.05	2.56 ± 0.07
(% Body weight)	3.8 ± 0.2 <sup>a</sup>	3.1 ± 0.1 <sup>b</sup>	3.5 ± 0.1 <sup>ab</sup>	3.7 ± 0.1 <sup>ab</sup>
Heart (g)	0.47 ± 0.03	0.47 ± 0.02	0.50 ± 0.02	0.42 ± 0.03
(% Body weight)	0.69 ± 0.03	0.62 ± 0.03	0.71 ± 0.03	0.59 ± 0.04
Uterus (g)	0.12 ± 0.04	0.13 ± 0.03	0.17 ± 0.03	0.20 ± 0.07
(% Body weight)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Testes (g)	0.07 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.06 ± 0.02
(% Body weight)	0.09 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.09 ± 0.00
Stomach (g)	0.54 ± 0.04	0.50 ± 0.03	0.54 ± 0.03	0.54 ± 0.05
(% Body weight)	0.77 ± 0.05	0.66 ± 0.04	0.76 ± 0.04	0.76 ± 0.06
S.intestine (g)	1.79 ± 0.1	1.94 ± 0.1	1.73 ± 0.1	1.82 ± 0.1
(% Body weight)	2.6 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.1

L.intestine (g)	0.73 ± 0.06	0.83 ± 0.05	0.86 ± 0.05	1.06 ± 0.2
(% Body weight)	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.5 ± 0.2
Caecum (g)	0.37 ± 0.03 <sup>a</sup>	0.46 ± 0.02 <sup>b</sup>	0.40 ± 0.02 <sup>ab</sup>	0.39 ± 0.03 <sup>ab</sup>
(% Body weight)	0.56 ± 0.03	0.59 ± 0.02	0.54 ± 0.02	0.6 ± 0.03
Total GI tract (g)	3.33 ± 0.14 <sup>a</sup>	3.78 ± 0.11 <sup>b</sup>	3.59 ± 0.10 <sup>ab</sup>	3.82 ± 0.20 <sup>b</sup>
(% Body weight)	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	5.4 ± 0.2
M.soleus (g)	0.027 ± 0.003	0.027 ± 0.002	0.024 ± 0.002	0.027 ± 0.004
(% Body weight)	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
M.gastroc (g)	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
(% Body weight)	0.19 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
M.biceps (g)	0.04 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.04
(% Body weight)	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00
Triceps (g)	0.17 ± 0.01 <sup>a</sup>	0.22 ± 0.01 <sup>b</sup>	0.21 ± 0.01 <sup>b</sup>	0.20 ± 0.01 <sup>ab</sup>
(% Body weight)	0.25 ± 0.01 <sup>a</sup>	0.29 ± 0.008 <sup>b</sup>	0.29 ± 0.007 <sup>b</sup>	0.28 ± 0.009 <sup>ab</sup>
Total muscle (g)	0.36 ± 0.21 <sup>a</sup>	0.44 ± 0.16 <sup>b</sup>	0.43 ± 0.15 <sup>b</sup>	0.40 ± 0.02 <sup>ab</sup>
(% Body weight)	0.56 ± 0.02	0.57 ± 0.02	0.55 ± 0.01	0.56 ± 0.02
Perirenal fat (g)	0.81 ± 0.03 <sup>a</sup>	0.85 ± 0.03 <sup>ab</sup>	0.83 ± 0.03 <sup>ab</sup>	0.97 ± 0.05 <sup>b</sup>
(% Body weight)	1.2 ± 0.03 <sup>b</sup>	1.1 ± 0.03 <sup>b</sup>	1.2 ± 0.03 <sup>b</sup>	1.4 ± 0.06 <sup>b</sup>
Interscap fat (g)	1.03 ± 0.05 <sup>a</sup>	1.09 ± 0.04 <sup>ab</sup>	1.06 ± 0.04 <sup>a</sup>	1.27 ± 0.07 <sup>b</sup>
(% Body weight)	1.5 ± 0.06 <sup>a</sup>	1.4 ± 0.05 <sup>a</sup>	1.4 ± 0.05 <sup>a</sup>	1.8 ± 0.1 <sup>b</sup>
Retro fat (g)	0.63 ± 0.04 <sup>a</sup>	0.78 ± 0.03 <sup>b</sup>	0.74 ± 0.03 <sup>b</sup>	0.76 ± 0.04 <sup>b</sup>
(% Body weight)	0.9 ± 0.04	1.0 ± 0.03	1.0 ± 0.03	1.1 ± 0.04 <sup>b</sup>
Total fat (g)	2.39 ± 0.11 <sup>a</sup>	2.77 ± 0.09 <sup>b</sup>	2.72 ± 0.08 <sup>ab</sup>	3.0 ± 0.1 <sup>b</sup>
(% Body weight)	3.6 ± 0.1 <sup>a</sup>	3.6 ± 0.1 <sup>a</sup>	3.7 ± 0.09 <sup>a</sup>	4.3 ± 0.1 <sup>b</sup>
Carcass (g)	48.68 ± 2.0 <sup>a</sup>	58.01 ± 1.6 <sup>b</sup>	53.98 ± 1.5 <sup>ab</sup>	53.00 ± 2.2 <sup>ab</sup>
(% Body weight)	73 ± 0.8	75 ± 0.6	74 ± 0.6	74 ± 0.8

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Different superscripts denote significant difference between groups, a vs b,  $P < 0.05$ . Statistical analyses were performed using Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject, the fetus as the repeated measure and the number of viable pups as the covariate. Abdominal circ = Abdominal circumference, Gastro = gastrocnemius, Interscap = interscapular fat, L.intestine = large intestine, Retro fat = retroperitoneal fat, S.intestine = small intestine.

### 5.3 Discussion

The present study demonstrates for the first time that administration of IGF-II to the mother in early to mid pregnancy increases placental structural and functional capacity by increasing the volume and surface area of the exchange region of the placenta near term, while IGF-I has no effect on the placenta. IGF-I, in contrast, reduced maternal adiposity late in pregnancy, while IGF-II did not affect maternal body composition. Importantly, however, maternal treatment with either IGF in early to mid pregnancy substantially reduced fetal resorptions and increased fetal viability and weight near term. This suggests that maternal IGF abundance, particularly that of IGF-II, during the period of early placental growth and development may in part determine the margin of safety between placental capacity to deliver, and fetal demand for, substrates throughout pregnancy. Importantly, administration of Leu<sup>27</sup>-IGF-II, an analogue of IGF-II with markedly reduced affinity for IGF1R and InsR, suggest that the impacts of IGF-II on the fetus are secondary to effects on the placenta, which are mediate predominantly through IGF2R.

In the current study, administration of 1mg/kg/day IGFs has been demonstrated to increase the abundance of bio-available maternal circulating IGF-II and IGF-I by 2.4 to 3.4-fold, during early to mid pregnancy (Chapter 4, p 109). Similar IGF treatment of guinea pigs during early to mid-pregnancy increased placental weight at mid-gestation (Sohlstrom, Fernberg *et al.* 2001), which was not sustained to near term in the current study. Importantly, however, the functional capacity of the placenta, as indicated by the mid-sagittal cross-sectional area, proportion and volume of the region devoted to exchange (labyrinth) were increased late in gestation, by prior maternal IGF-II treatment. Furthermore, although the composition of the exchange region of the placenta was unaltered by earlier maternal IGF treatment, the total volume of trophoblast and maternal blood spaces, as well as the total surface area of placenta functioning in exchange, were increased by IGF-II. As the labyrinth expands at the expense of the interlobium in the second half of pregnancy in the guinea pig (Kaufmann and Davidoff 1977; Roberts, Sohlstrom *et al.* 2001a), together these changes placental structure following earlier exogenous maternal IGF-II, are suggestive of a more mature placenta and would be expected to increase placental transport capacity. In contrast, maternal exogenous IGF-I had no effect on structural correlates of placental function.

Rapid placental structural differentiation and growth occurs in early to mid gestation in all eutherian mammals. In humans and guinea pigs, trophoblasts invade deeply within the uterus and its arterioles, extensively remodelling them, to permit increased maternal blood flow to the placenta (Hees, Moll *et al.*

1987; Lyall 2005; Moll, Espach *et al.* 1983; Pijnenborg, Bland *et al.* 1981; Pijnenborg, Robertson *et al.* 1981; Pijnenborg, Vercruysse *et al.* 2006). This ensures delivery of oxygen and nutrients to the placenta, and subsequently to the fetus. The sustained effects of maternal IGF-II supplementation in early to mid pregnancy on the placenta reported here are the converse of those observed following specific deletion of IGF-II within the placenta. IGF-II is abundantly expressed by invasive trophoblasts of human (Han, Bassett *et al.* 1996), mouse (Redline, Chernicky *et al.* 1993), rat (Zhou and Bondy 1992) and guinea pig placenta (Han, Carter *et al.* 1999). Ablation of placenta specific *Igf2* gene expression (P0 transcript) in mice reduced the surface area for exchange, increased the exchange barrier thickness and also impaired nutrient transport capacity of the placenta (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004).

Reduced maternal circulating IGF-II in mid-pregnancy, as a result of under-nutrition in guinea pigs (Sohlstrom, Katsman *et al.* 1998b), is associated with similar consequences to those of placental *Igf2* gene deletion (Sibley, Coan *et al.* 2004), with a delay and impairment in the functional maturation of the placenta and with reduced fetal growth in both mid and late gestation (Roberts, Sohlstrom *et al.* 2001b). Together these findings indicate that maternal circulating IGF-II may act in an endocrine fashion to modulate placental development, in addition to any autocrine / paracrine effects of locally produced IGF-II. We suggest that exposure to increased circulating maternal IGF-II in early to mid pregnancy may provide a foundation of increased placental trophoblast proliferation and invasion of the uterus and its vasculature, which leads to increased volumes of both trophoblast and maternal blood spaces in the placental labyrinth in late gestation. This would be expected to increase maternal blood flow to the placenta and enhance growth of the area devoted to exchange improving placental transfer of oxygen and nutrients to the fetus from the mother. Hence, maternal IGF-II supplementation presumably increased fetal growth and viability predominantly by these actions on the placenta.

Supplementing the mother during early to mid pregnancy with either IGF had a sustained positive effect on fetal weight, length and girth near term, which is consistent with the anabolic effects on the fetus seen at mid-pregnancy following similar treatment in the guinea pig (Sohlstrom, Fernberg *et al.* 2001). The increased fetal weight observed with maternal IGF treatment appears to be substantially due to increased muscle mass overall and proportionately for selected muscles, and perhaps enhanced fetal bone growth as indicated by increased carcass weights. This may be metabolically beneficial in later life because muscle is an important site for insulin-induced glucose uptake. Indeed, fetal growth restriction in the guinea pig, induced by maternal food restriction and accompanied by reductions in circulating maternal IGF concentrations (Sohlstrom, Katsman *et al.* 1998b), is characterised by deficits in muscle

mass, increased adiposity in the fetus near term (Kind, Roberts *et al.* 2005) and with increased blood pressure and impaired glucose and cholesterol homeostasis in adult offspring (Kind, Clifton *et al.* 2003; Kind, Clifton *et al.* 1999; Kind, Simonetta *et al.* 2002).

The present study suggests that increased maternal IGF-I and IGF-II abundances during early to mid pregnancy, promote fetal growth and viability near term by multiple mechanisms. In addition to direct effects of IGF-II on placental structural development, which in the current study were positively associated with fetal weight, the IGFs may increase nutrient transporter expression (Bloxam, Bax *et al.* 1997; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998) and/or placental vasodilation (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995), which would allow for more substrate to be delivered to the fetus for its growth. The IGFs may also influence placental metabolism and function, which, in turn may drive major physiological adaptations to pregnancy in the mother, including the development of insulin resistance to divert nutrients to the conceptus (Butte 2000; Catalano, Drago *et al.* 1995; Catalano and Hollenbeck 1992). This has been attributed to placental production of hormones including estrogen, progesterone and placental lactogen (Butte 2000; Ryan and Enns 1988) that reduce maternal insulin secretion (Butte 2000; Picard, Wanatabe *et al.* 2002) and antagonise the effects of insulin on maternal tissues, including fat deposition (Ryan and Enns 1988). Treatment of the mother with IGF-II enhanced placental weight in mid-pregnancy (Sohlstrom, Fernberg *et al.* 2001) and was accompanied by elevated maternal circulating estradiol and progesterone concentrations (Chapter 4, p 122), although these were not significant. This would be expected to amplify insulin resistance and other adaptations such as fat deposition in the mother. Consistent with this, exogenous IGF-II during early to mid pregnancy in guinea pigs increased maternal interscapular adiposity at mid-pregnancy (Sohlstrom, Fernberg *et al.* 2001) near term. These increased maternal adipose stores were restored to normal by late pregnancy in the current study, which may have further enhanced nutrient availability for the fetus, either directly or indirectly. This suggests that IGF-II acts on the placenta to increase fetal growth, by sustainably promoting placental development but may additionally enhance maternal physiological adaptation to pregnancy.

The mechanism by which increased maternal IGF-I abundance in early to mid pregnancy sustainably promotes fetal growth is less clear. The enhanced placental weight at mid gestation by prior maternal IGF-I treatment (Sohlstrom, Fernberg *et al.* 2001) (Chapter 4, p 112), which is no longer apparent in late gestation, may have had persistent effects on the fetus that increased fetal growth near term. In addition, unlike IGF-II, IGF-I did not increase maternal fat deposition in mid pregnancy (Sohlstrom, Fernberg *et al.* 2001) and in fact reduced fat depot weights near term. This is consistent with the lipolytic

effect of administered IGF-I in non-pregnant sheep (Lewis, Molan *et al.* 1988). Reduced adiposity may reflect increased mobilization and/or reduced deposition during pregnancy, which may have increased substrate availability in the maternal circulation for fetal growth. This has been observed in growth hormone treated pigs, where maternal circulating IGF-I concentration was elevated and associated with reductions in maternal backfat depots (Gatford, Owens *et al.* 2000). Another possible explanation is that larger fetuses of IGF-I treated dams may signal to the mother via nutrient sensors in the fetal circulation (such as IGFs and insulin), to influence placental metabolism and increase mobilisation of maternal adipose tissue stores late in pregnancy.

These different IGF effects may reflect their distinct interactions with various receptors, since IGF-I binds with high affinity to the IGF1R but negligibly to IGF2R. In contrast, IGF-II binds to both these receptors, as well as to the insulin receptor (InsR). The guinea pig placenta ubiquitously expressed both IGF receptor proteins in mid pregnancy (Chapter 4, p 109). More importantly, however, at the time of IGF treatment, IGF1R and IGF2R were localised to the apical surface of trophoblasts, within large maternal blood vessels and blood spaces of the labyrinth permitting endocrine and exogenous IGFs to act directly within the placenta. In addition, insulin binding sites have previously been identified in trophoblast of the guinea pig placenta (Kelly, Posner *et al.* 1974; Posner 1974). This pattern of expression is consistent with the localisation of all three receptors to placental trophoblasts in humans and rats (Abu-Amero, Ali *et al.* 1998; Fang, Furesz *et al.* 1997; Jones, Hartmann *et al.* 1993; Korgun, Dohr *et al.* 2003; Millio, Hu *et al.* 1994; Ohlsson, Holmgren *et al.* 1989; Zhou and Bondy 1992) and abundant expression of IGF1R and IGF2R in invasive trophoblast populations within the human decidua and its vasculature (Fang, Furesz *et al.* 1997).

The specific effects of IGF-II on the placenta, which were not evident in IGF-I treated animals, suggest that IGF-II actions on the placenta may be mediated by the InsR, which has been implicated in mediating IGF-II effects on fetal growth (Louvi, Accili *et al.* 1997) or by the IGF2R, which it binds with much greater affinity than the IGF1R (Jones and Clemmons 1995). In the current study we have attempted to discriminate the contribution of IGF2R in mediating IGF-II effects on placental development and fetal growth using Leu<sup>27</sup>-IGF-II, an analogue of IGF-II that has significantly reduced binding capacity for the IGF1R and InsR (80 and 220-fold lower affinity, respectively (Sakano, Enjoh *et al.* 1991) and only slightly decreased affinity for the IGF binding proteins (Bach, Hsieh *et al.* 1993). Unexpectedly, placental weight was different between Leu<sup>27</sup>-IGF-II and IGF-II treated mothers. However, there was no effect of treatment on placental weight compared with vehicle treated controls. Importantly, analysis of placental



structure revealed that many of the effects seen on placental labyrinthine development with maternal IGF-II treatment, were mirrored by Leu<sup>27</sup>-IGF-II administration. Like IGF-II, Leu<sup>27</sup>-IGF-II increased labyrinth proportion and volume, while reducing the weight of the interlobium. It certainly seemed that Leu<sup>27</sup>-IGF-II was more potent than IGF-II at promoting labyrinthine development, as seen by increased volume densities of trophoblast and maternal blood spaces, increased total surface area of trophoblast for exchange and reduction in the thickness of the barrier for diffusion. This is consistent with effects of IGF-II being mediated through IGF2R to promote trophoblast migration and invasion (McKinnon, Chakarborty *et al.* 2000), and uterine angiogenesis and vascular remodelling (Herr, Liang *et al.* 2003).

In contrast, the effects of maternal IGF-I treatment are likely to have been mediated by the IGF1R, particularly since this treatment also reduced IGF-II in the mother. Interestingly, the effects of IGF-I on maternal and fetal body composition were similar to those seen in Leu<sup>27</sup>-IGF-II, but not IGF-II treated mothers. For instance, mothers that had been treated with Leu<sup>27</sup>-IGF-II during early to mid pregnancy, also displayed reduced retroperitoneal and interscapular adiposity near term. This may have been indirectly due to an increased interaction of endogenous maternal circulating IGF-II with IGF1R in the Leu<sup>27</sup>-IGF-II treated animals because of the selective interaction of Leu<sup>27</sup>-IGF-II with IGF2R. Other IGF analogues with greater specificity for the IGF1R and InsR may be helpful in elucidating these effects further.

In conclusion, increased maternal IGF-II in early pregnancy sustainably promotes placental structural and functional capacity and fetal growth and viability, while IGF-I appears to act through the mother to enhance fetal growth to near term. This suggests sustained major and complementary roles in placental and fetal growth for increased circulating IGFs in the mother in early pregnancy.

*Chapter 6 : Sustained effects of  
maternal IGF treatment on placental  
transport and nutrient partitioning  
between the mother and fetus*

## 6.1 Introduction

The relationship between maternal intake and utilization of substrates during pregnancy and their supply to the conceptus determines pregnancy success and life-long health of offspring (Redmer, Wallace *et al.* 2004). Adequate nutrients must be delivered to the growing placenta and fetus, as well as to the mother, to meet the energy requirements needed to maintain maternal health and her capacity to support the conceptus. To ensure the latter during normal pregnancy, the mother undergoes a number of physiological changes in her appetite, body composition, cardiovascular function, energy consumption and metabolism (Owens 1991). The placenta is central to these processes, as it is not only the organ responsible for the exchange of substrates between the mother and fetus, but also synthesises a number of steroid and peptide hormones, secreted into the maternal circulation, that modulate maternal physiology and adaptation to pregnancy.

Impaired supply of nutrients to the fetus causes intrauterine growth restriction (IUGR) (Cetin, Corbetta *et al.* 1990; Cetin, Radaelli *et al.* 2001), which currently affects 6% of Australian births (Laws and Sullivan 2004) and up to 40% in developing countries (Albertsson-Wikland, Wennergren *et al.* 1993). IUGR is associated with perinatal morbidity and mortality (Fitzhardinge 1985; Low, Handley-Derry *et al.* 1992) and increases the risk of poor health in childhood and adult life (Barker 1998). Understanding the factors essential for regulating nutrient partitioning during pregnancy may help to identify the causes of IUGR and possibly the development of novel therapeutics.

The insulin-like growth factors (IGFs) are implicated as major factors influencing nutrient partitioning between the mother and fetus. Substantial pregnancy associated changes in maternal circulating IGF-I and IGF-II occur in several species (Donovan, Giudice *et al.* 1991; Gargosky, Moyse *et al.* 1990; Hills, English *et al.* 1996; Nason, Binder *et al.* 1996; Sohlstrom, Katsman *et al.* 1998b; Travers, Madon *et al.* 1990; Wilson, Bennett *et al.* 1982), with IGF-II, in particular, also highly expressed in the placenta (Han and Carter 2000). While maternal IGFs do not cross the placenta in physiologically significant quantities (Brown and Thorburn 1989), they may act on the placenta and maternal tissues to regulate nutrient allocation between the mother, placenta and fetus in various ways (Wallace, Da Silva *et al.* 1997).

Maternal IGF-I may improve or maintain nutrient transfer to the fetus by enhancing placental transport and modification of nutrients or by increasing substrate availability in the mother for transfer to the fetus, as seen in the pregnant ewe (Harding, Liu *et al.* 1994). This may also be the case in women, as IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (Bloxam, Bax *et al.*

1994; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998). Furthermore, IGF-I inhibits the release of vasoconstrictors in term human placental villous explants (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995), which may increase placental blood flow and delivery of nutrients during pregnancy. However, there is also evidence to suggest that IGF-I may promote maternal anabolism over fetal growth. IGF-I administration in the second half of pregnancy increased maternal weight gain near term but did not alter fetal and placental growth in rats (Gargosky, Owens *et al.* 1991). In addition, elevated maternal circulating IGF-I induced by over nourishing the singleton-bearing adolescent sheep, correlated with increased maternal tissue accretion at the expense of the fetus and placenta (Wallace, Aitken *et al.* 1996; Wallace, Da Silva *et al.* 1997).

IGF-II may also act locally to modulate placental development and transport function as occurs in mice. Indeed, placental amino acid transporter expression is altered by *Igf2* deficiency in mice (Matthews, Beveridge *et al.* 1999) and ablation of the trophoblast specific *Igf2* promoter (*P0*), reduces placental weight and adversely affects placental structural differentiation and transport capacity (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004). In the *Igf2 P0* mutant placenta, passive diffusion of inert hydrophilic solutes is reduced, while System A amino acid (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004) and glucose (Constancia, Angiolini *et al.* 2005) transport are transiently up-regulated, apparently via direct impacts on placental transporter gene expression (Constancia, Angiolini *et al.* 2005).

In guinea pigs and humans, maternal circulating concentrations of IGFs are substantial (Gargosky, Moyse *et al.* 1990; Sohlstrom, Katsman *et al.* 1998b) and may have major influences on nutrient partitioning during pregnancy. Indeed, we have shown in the guinea pig that increasing concentrations of IGF by infusion of IGF-I or IGF-II into the mother during early to mid pregnancy enhanced fetal growth (largely due to increased muscle mass) and survival near term, but possibly in part via different mechanisms (Chapter 5). In the IGF-I treated mother, the nutritional requirements of the conceptus appeared to take priority over those of the mother, as maternal adipose stores were depleted in late pregnancy (without altering placental development), possibly increasing substrate availability for the conceptus (Chapter 5). In contrast, IGF-II treatment in early to mid pregnancy did not affect maternal body composition but enhanced development of the placental exchange region in late gestation, suggestive of improved placental functional capacity (Chapter 5). Additionally, IGF supplementation increased concentrations of steroid hormones in the maternal circulation, which may have modified maternal physiological responses to pregnancy and, in turn, nutrient partitioning (Chapter 5).

The aim of this study was to dissect the mechanisms underlying maternal IGF influences in early to mid pregnancy on substrate availability to, and hence growth of, the fetus near term. To achieve this, we measured placental uptake and transfer of non-metabolisable radioanalogues of glucose ( $[^3\text{H}]$ -methyl-D-glucose, MG) and amino acids ( $[^{14}\text{C}]$ -amino-isobutyric acid, AIB), as well as circulating metabolite concentrations in the fetus and mother, on day 62 of pregnancy, just prior to term (term = 69 days). In light of our previous report of differences in fetal and maternal body composition near term in response to earlier maternal IGF treatment (Chapter 5), we also determined MG and AIB uptake by several fetal and maternal tissues. Furthermore, due to differences in maternal body composition between IGF-I and IGF-II treatment, we also aimed to elucidate the role of type 2 IGF receptor (IGF2R) in mediating IGF-II influences on nutrient uptake during pregnancy by maternal administration of Leu<sup>27</sup>-IGF-II, an IGF-II analogue selective for IGF2R (Sakano, Enjoh *et al.* 1991).

## 6.2 Results

### *6.2.1.1 Sustained effect of exogenous maternal IGF treatment on litter composition and fetal and placental weights*

Some of the data in this chapter were common to those in chapter 5. Data in this chapter has been restricted to animals used in placental transport studies. Litters from mothers that had been treated with IGF-I and Leu<sup>27</sup>-IGF-II during early to mid pregnancy had a greater number of viable fetuses (+30%,  $P = 0.001$ ; +40%,  $P = 0.002$ , respectively) and fewer resorptions (-84%,  $P = 0.007$ ; -100%,  $P = 0.01$ , respectively) compared with vehicle treated mothers, near term (Table 6.1). However, total litter size was unaffected by earlier maternal IGF treatment (Table 6.1). Maternal IGF-I treatment increased fetal weight near term (+10%,  $P = 0.05$ ) but there was no effect of treatment on placental weight (Table 6.1).

### *6.2.1.2 Sustained effects of exogenous maternal IGF on placental uptake of methyl glucose and amino isobutyric acid*

Treating the mother with IGF-I during early to mid pregnancy increased placental MG uptake compared to vehicle treated mothers near term in per gram and total placenta terms (+78%,  $P = 0.001$  and +70%,  $P = 0.004$ , respectively) (Figure 6.1a). Placentae from mothers treated with IGF-I had higher MG uptake per gram of placenta compared with those treated with IGF-II ( $P = 0.019$ ).

Earlier maternal IGF treatment did not alter placental uptake of AIB on a per gram of tissue or whole tissue basis (Figure 6.1b). Leu<sup>27</sup>-IGF-II increased AIB uptake per gram of placenta, compared with vehicle and IGF-II treated animals (+65%,  $P = 0.05$ ; +46%,  $P = 0.058$ , respectively), without an alteration in total placental AIB uptake (Figure 6.1b).

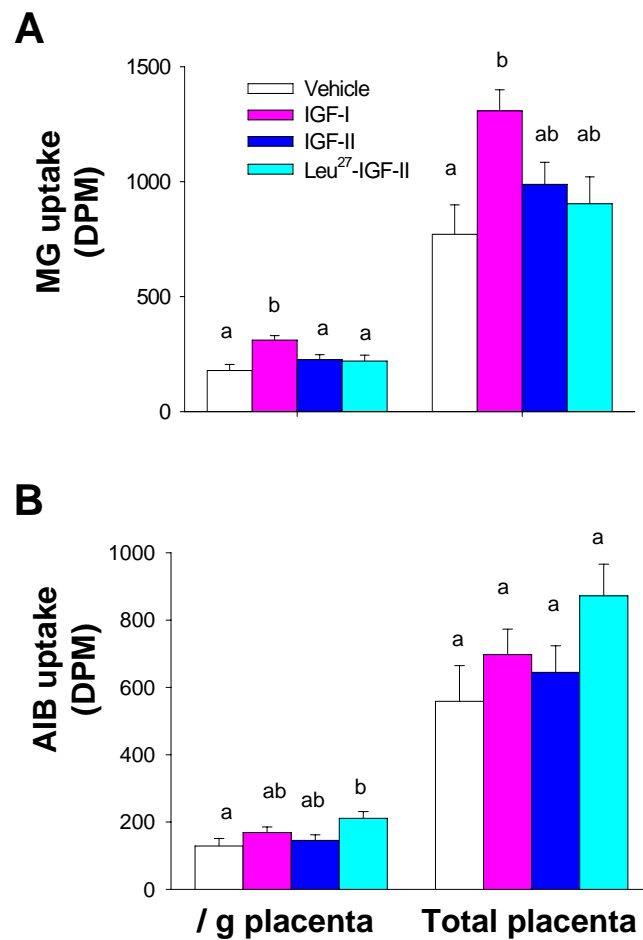
### *6.2.1.3 Sustained effects of exogenous maternal IGF on fetal plasma methyl glucose and amino isobutyric acid concentrations*

Maternal IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treatment during early to mid pregnancy increased the concentration of fetal plasma MG near term (+50%,  $P = 0.002$ ; +41%,  $P = 0.012$ ; +89%,  $P < 0.0001$ , respectively) (Figure 6.2a). Additionally, exogenous maternal Leu<sup>27</sup>-IGF-II increased the concentration

**Table 6.1 Sustained effect of maternal IGF treatment in early to mid pregnancy on litter composition and fetal dimensions**

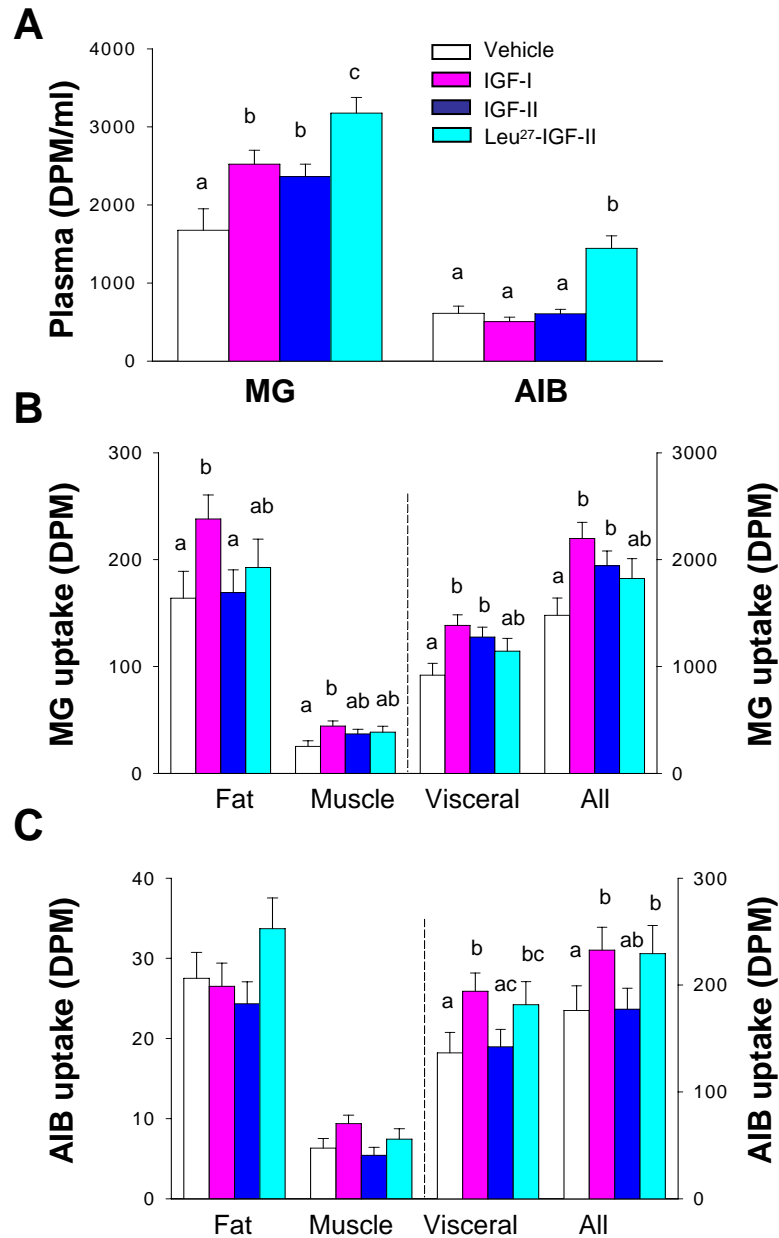
	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Dams	8	9	6	4
Fetuses	20	30	18	13
Female / Male	9 / 10	12 / 10	14 / 16	9 / 4
Total Litter	3.1 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.5 ± 0.2
Number viable	2.5 ± 0.2 <sup>a</sup>	3.3 ± 0.1 <sup>b</sup>	3.0 ± 0.2 <sup>ab</sup>	3.5 ± 0.2 <sup>b</sup>
Number resorbing	0.61 ± 0.1 <sup>a</sup>	0.10 ± 0.01 <sup>b</sup>	0.25 ± 0.1 <sup>ab</sup>	0.0 ± 0.0 <sup>b</sup>
Fetal weight <sup>**</sup>	69.7 ± 2.4 <sup>a</sup>	76.8 ± 1.9 <sup>b</sup>	71.1 ± 2.6 <sup>ab</sup>	72.0 ± 2.8 <sup>ab</sup>
Placenta weight <sup>**</sup>	4.26 ± 0.2	4.26 ± 0.1	4.28 ± 0.2	4.07 ± 0.2

Data are expressed as mean ± SEM or <sup>\*\*</sup> estimated marginal mean ± SEM after adjusting for the number of viable pups per litter. Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject, the fetus as the repeated measure and the number of viable pups as the covariate (when required). Different superscripts denote significant differences between groups, a vs b P < 0.05.



**Figure 6.1** Sustained effect of maternal IGF treatment in early to mid pregnancy on placental tissue uptake of substrate. Placental uptake of [<sup>3</sup>H]-methyl-D-glucose (MG, **a**) and [<sup>14</sup>C]-aminoisobutyric acid (AIB, **b**) per gram and total tissue. Data are from all placentae obtained from mothers treated with vehicle (mothers = 6), IGF-I (mothers = 7), IGF-II (mothers = 6) or Leu<sup>27</sup>-IGF-II (mothers = 4). Values are expressed as estimated marginal mean DPM ± SEM. Different superscripts denote a statistically significant difference between treatments, a vs b P < 0.05, as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject, the fetus as the repeated measure and the number of viable pups per litter as the covariate.





**Figure 6.2 Sustained effect of maternal IGF treatment in early to mid pregnancy on fetal substrate utilization.** Fetal concentrations of [<sup>3</sup>H]-methyl-D-glucose (MG) and [<sup>14</sup>C]-aminoisobutyric acid (AIB) in plasma (a) and tissues (c and d). Values are expressed as estimated marginal mean DPM / ml ± SEM (a) and estimated marginal mean total DPM ± SEM (c and d). Data are from all fetuses obtained from mothers treated with vehicle (mothers = 6), IGF-I (mothers = 7), IGF-II (mothers = 6) or Leu<sup>27</sup>-IGF-II (mothers = 4). Different superscripts denote statistically significant difference,  $P < 0.048$ , as determined by a Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject, the fetus as the repeated measure and the number of viable pups per litter as the covariate.

of AIB in fetal plasma near term, compared with vehicle (+240%,  $P = 0.024$ ), IGF-I (+290%,  $P < 0.0001$ ) and IGF-II (+238%,  $P < 0.0001$ ) treatment groups (Figure 6.2a).

Across all treatments, fetal plasma MG was positively correlated with fetal weight ( $r = 0.29$ ,  $P = 0.031$ ) and crown-rump length ( $r = 0.28$ ,  $P = 0.036$ ). Across all treatments, placental MG uptake per gram of tissue and total uptake was positively correlated with fetal plasma MG concentrations ( $r = 0.92$ ,  $P < 0.001$  and  $r = 0.92$ ,  $P < 0.0001$ ). Total placental uptake of MG was additionally positively associated with fetal weight ( $r = 0.91$ ,  $P < 0.0001$ ).

#### *6.2.1.4 Sustained effects of exogenous maternal IGF treatment on fetal tissue methyl glucose uptake*

Early to mid pregnancy maternal IGF-I increased MG uptake per gram by fetal spleen (+42%,  $P = 0.05$ ) perirenal fat (+49%,  $P = 0.043$ ), lung (+63%,  $P = 0.037$ ), liver (+36%,  $P = 0.037$ ) and kidney (+51%,  $P = 0.001$ ) and total MG uptake of retroperitoneal fat (+74%,  $P = 0.002$ ), perirenal fat (+85%,  $P = 0.003$ ), lung (+84%,  $P = 0.013$ ), liver (+49%,  $P = 0.041$ ), kidney (+81%,  $P < 0.001$ ), heart (+88%,  $P = 0.043$ ), gastrocnemius (+99%,  $P = 0.015$ ) and triceps (+77%,  $P = 0.036$ ) compared to vehicle near term (Table 6.2).

Early to mid pregnancy maternal IGF-II increased MG uptake per gram by fetal lung (+50%,  $P = 0.043$ ) and liver (+34%,  $P = 0.037$ ) and total MG uptake by lung (+52%,  $P = 0.016$ ), liver (+43%,  $P = 0.05$ ), gastrocnemius (+49%,  $P = 0.049$ ) and triceps (+44%,  $P = 0.05$ ) compared to vehicle near term (Table 6.2). Early to mid pregnancy treatment of the mother with Leu<sup>27</sup>-IGF-II increased total MG uptake (DPM) by fetal triceps (+69%,  $P = 0.045$ ) (Table 6.2).

MG uptake by the fetal kidney in total (DPM) and relative per gram terms were 23% and 39% greater with maternal IGF-I treatment compared with IGF-II, respectively ( $P = 0.049$  and  $P = 0.018$ ). Total MG uptake by the fetal retroperitoneal fat was 42% greater following maternal IGF-I treatment compared with IGF-II ( $P = 0.025$ ) (Table 6.2). For all treatment groups, the uptake of MG per gram of tissue was greatest in the fetal kidney and lowest in the retroperitoneal fat, except for the Leu<sup>27</sup>-IGF-II animals where uptake was lowest in the interscapular fat (Table 6.2).

**Table 6.2 Sustained effect of maternal IGF treatment in early to mid pregnancy on fetal tissue methyl glucose uptake**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number mums	6	7	6	4
Number of fetuses	17	18	16	13
Brain (DPM / gram)	150 ± 20	191 ± 18	179 ± 17	176 ± 28
(total DPM)	369 ± 49	496 ± 45	462 ± 42	449 ± 69
Heart (DPM / gram)	147 ± 30	220 ± 25	176 ± 24	177 ± 29
(total DPM)	62 ± 16 <sup>a</sup>	116 ± 14 <sup>b</sup>	84 ± 13 <sup>ab</sup>	78 ± 16 <sup>ab</sup>
Liver (DPM / gram)	154 ± 17 <sup>a</sup>	209 ± 17 <sup>b</sup>	206 ± 14 <sup>b</sup>	184 ± 18 <sup>ab</sup>
(total DPM)	559 ± 82 <sup>a</sup>	833 ± 81 <sup>b</sup>	799 ± 69 <sup>b</sup>	687 ± 89 <sup>ab</sup>
Lungs (DPM / gram)	118 ± 21 <sup>a</sup>	192 ± 20 <sup>b</sup>	177 ± 18 <sup>b</sup>	140 ± 22 <sup>ab</sup>
(total DPM)	159 ± 33 <sup>a</sup>	293 ± 30 <sup>b</sup>	241 ± 28 <sup>b</sup>	197 ± 34 <sup>ab</sup>
Kidneys (DPM / gram)	187 ± 17 <sup>a</sup>	282 ± 16 <sup>b</sup>	229 ± 14 <sup>a</sup>	234 ± 19 <sup>ab</sup>
(total DPM)	102 ± 14 <sup>a</sup>	184 ± 13 <sup>b</sup>	132 ± 12 <sup>a</sup>	137 ± 16 <sup>ab</sup>
Spleen (DPM / gram)	115 ± 15 <sup>a</sup>	164 ± 13 <sup>b</sup>	147 ± 13 <sup>ab</sup>	133 ± 15 <sup>ab</sup>
(total DPM)	18 ± 3	19 ± 3	15 ± 3	13 ± 3
M.Tric (DPM / gram)	72 ± 11	102 ± 12	98 ± 10	110 ± 13
(total DPM)	13 ± 3 <sup>a</sup>	23 ± 3 <sup>b</sup>	18 ± 2 <sup>b</sup>	22 ± 3 <sup>b</sup>
M. Gast (DPM / gram)	101 ± 17	154 ± 15	128 ± 14	124 ± 17
(total DPM)	12 ± 3 <sup>a</sup>	25 ± 3 <sup>b</sup>	19 ± 2 <sup>b</sup>	17 ± 3 <sup>ab</sup>
Inters (DPM / gram)	94 ± 23	78 ± 22	66 ± 19	56 ± 22
(total DPM)	84 ± 19	80 ± 18	69 ± 16	70 ± 18
Periren (DPM / gram)	65 ± 10 <sup>a</sup>	97 ± 9 <sup>b</sup>	71 ± 8 <sup>ab</sup>	75 ± 10 <sup>ab</sup>
(total DPM)	45 ± 9 <sup>a</sup>	86 ± 8 <sup>b</sup>	58 ± 7 <sup>a</sup>	75 ± 10 <sup>ab</sup>
Retro (DPM / gram)	55 ± 8	76 ± 7	62 ± 7	67 ± 8
(total DPM)	35 ± 5 <sup>a</sup>	61 ± 5 <sup>b</sup>	43 ± 4 <sup>a</sup>	47 ± 6 <sup>ab</sup>

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. M.Gast, gastrocnemius; M.Tric, triceps, Inters, interscapular fat; Periren, perirenal fat; Retro, retroperitoneal fat. Superscripts denote significant difference between groups, a vs b,  $P < 0.05$ , as determined by Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject, the fetus as the repeated measure and the number of viable pups as the covariate.

Maternal IGF-I treatment increased MG uptake per gram by fetal visceral tissues by 38% (mean DPM / gram  $\pm$  SEM; vehicle:  $735 \pm 69$ , IGF-I:  $1016 \pm 65$ ; IGF-II:  $936 \pm 61$ , Leu<sup>27</sup>-IGF-II:  $857 \pm 77$ ,  $P = 0.014$ ) and combined internal organs and tissues by 34% (mean DPM / gram  $\pm$  SEM; vehicle:  $1271 \pm 132$ , IGF-I:  $1705 \pm 118$ ; IGF-II:  $1540 \pm 111$ , Leu<sup>27</sup>-IGF-II:  $1495 \pm 141$ ,  $P = 0.05$ ). Maternal IGF-I treatment increased total MG uptake by fetal fat (+45%,  $P = 0.046$ ) and muscle (76%,  $P = 0.027$ ) and IGF-I and IGF-II increased total MG uptake by fetal visceral tissues (+51%,  $P = 0.009$  and 39%,  $P = 0.05$  respectively) and combined internal organs and tissues (+49%,  $P = 0.007$ , +31%,  $P = 0.048$ ) (Figure 6.2b). There was no significant effect of Leu<sup>27</sup>-IGF-II on combined fetal tissue uptake of MG (total DPM). Across all treatments, placental MG uptake was positively correlated with fetal plasma and tissue MG counts (data not shown).

Placental MG transfer capacity (total fetal tissue counts divided by placental weight) was increased by earlier maternal IGF-I (+50%,  $P = 0.029$ ), IGF-II (+28%,  $P = 0.033$ ) or Leu<sup>27</sup>-IGF-II (+28%,  $P = 0.047$ ) treatments (mean  $\pm$  SEM; vehicle:  $349 \pm 43$ , IGF-I:  $506 \pm 39$ ; IGF-II:  $446 \pm 36$ , Leu<sup>27</sup>-IGF-II:  $446 \pm 46$ ).

#### *6.2.1.5 Sustained effects of exogenous maternal IGF on fetal tissue amino isobutyric acid uptake*

Compared to vehicle, maternal IGF-I and Leu<sup>27</sup>-IGF-II treatment during early to mid pregnancy increased AIB uptake per gram by the fetal heart (+56%,  $P = 0.033$ ; +61%,  $P = 0.048$ , respectively), which was also true in total uptake terms for IGF-I (+80%,  $P = 0.036$ ) (Table 6.3). Leu<sup>27</sup>-IGF-II also increased per gram and total uptake of AIB by the fetal lungs compared with vehicle (+73%,  $P = 0.031$ ; +80%,  $P = 0.02$ , respectively) and IGF-II (+58%,  $P = 0.044$ ; +69%,  $P = 0.027$ , respectively) treated animals (Table 6.3). There was no effect of maternal IGF-I or IGF-II treatment on AIB uptake by other fetal tissues (on a per gram or total tissue basis) (Table 6.3). The uptake of AIB per gram of tissue was lowest in the brain for all animals and greatest in the fetal liver in vehicle, IGF-I and IGF-II animals and the fetal kidney for Leu<sup>27</sup>-IGF-II (Table 6.3).

Maternal IGF-I treatment increased AIB uptake (DPM / gram) by fetal visceral tissues combined by 41% (mean DPM / gram  $\pm$  SEM; vehicle:  $96 \pm 12$ , IGF-I:  $135 \pm 12$ ; IGF-II:  $101 \pm 11$ ; Leu<sup>27</sup>-IGF-II:  $133 \pm 14$ ,  $P = 0.05$ ) and fetal muscle AIB by 59% compared with IGF-II (mean DPM / gram  $\pm$  SEM; vehicle:  $38 \pm 5.9$ , IGF-I:  $51 \pm 5.6$ ; IGF-II:  $32 \pm 5.3$ ; Leu<sup>27</sup>-IGF-II:  $44 \pm 6.9$ ,  $P = 0.05$ ). Maternal IGF-I treatment increased total AIB uptake into fetal visceral tissues compared with vehicle and IGF-II (+43%,  $P = 0.046$

**Table 6.3 Sustained effect of maternal IGF treatment in early to mid pregnancy on fetal tissue amino isobutyric acid uptake**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number mums	6	7	6	4
Number of fetuses	17	18	16	13
Brain (DPM / gram)	2.4 ± 0.4	2.7 ± 0.4	2.1 ± 0.4	2.6 ± 0.5
(total DPM)	6.2 ± 1.2	5.8 ± 0.9	5.4 ± 0.9	6.6 ± 1.2
Heart (DPM / gram)	18 ± 5 <sup>a</sup>	28 ± 3 <sup>b</sup>	21 ± 3 <sup>ab</sup>	29 ± 4 <sup>b</sup>
(total DPM)	7.8 ± 2.1 <sup>a</sup>	14.1 ± 1.6 <sup>b</sup>	9.7 ± 1.5 <sup>ab</sup>	13.1 ± 2.1 <sup>ab</sup>
Liver (DPM / gram)	29 ± 5	36 ± 4	28 ± 4	31 ± 5
(total DPM)	103 ± 17	135 ± 15	102 ± 13	116 ± 18
Lungs (DPM / gram)	11 ± 1.8 <sup>a</sup>	14 ± 1.4 <sup>ab</sup>	12 ± 1.4 <sup>a</sup>	19 ± 1.9 <sup>b</sup>
(total DPM)	15 ± 2.7 <sup>a</sup>	21 ± 2.7 <sup>ab</sup>	16 ± 2.1 <sup>a</sup>	27 ± 3 <sup>b</sup>
Kidneys (DPM / gram)	29 ± 5	29 ± 3	26 ± 3	36 ± 4
(total DPM)	16 ± 3	18 ± 2	13 ± 2	17 ± 3
Spleen (DPM / gram)	14 ± 4	18 ± 3	16 ± 3	20 ± 4
(total DPM)	2.1 ± 0.5	1.9 ± 0.3	1.7 ± 0.4	2.1 ± 0.4
M.Trice (DPM / gram)	18 ± 4	25 ± 3	15 ± 3	23 ± 4
(total DPM)	3.8 ± 0.9	5.6 ± 0.8	2.9 ± 0.7	4.6 ± 0.9
M. Gast (DPM / gram)	20 ± 4	23 ± 3	16 ± 3	21 ± 4
(total DPM)	2.7 ± 0.6	3.5 ± 0.4	2.3 ± 0.4	2.8 ± 0.6
Inters (DPM / gram)	13.5 ± 2.1	8.8 ± 1.7	8.6 ± 1.6	9.3 ± 2.1
(total DPM)	12.3 ± 2.0	9.3 ± 1.7	9.0 ± 1.5	11.6 ± 1.9
Periren (DPM / gram)	13 ± 1.8	13 ± 1.3	11 ± 1.4	15 ± 1.8
(total DPM)	8.9 ± 1.7	11.2 ± 1.3	8.9 ± 1.3	14.8 ± 1.7
Retro (DPM / gram)	9.7 ± 1.2	9.1 ± 0.9	8.3 ± 0.9	10.7 ± 1.2
(total DPM)	6.6 ± 1.0	7.2 ± 0.8	5.9 ± 0.8	7.6 ± 1.1

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. M.Gast, gastrocnemius; M.Trice, triceps, Inters, interscapular fat; Periren, perirenal fat; Retro, retroperitoneal fat. Superscripts denote significant difference between groups, a vs b, P < 0.05, as determined by Linear Mixed Model Repeated Measures with Sidak Post Hoc Tests, using the mother as the subject, the fetus as the repeated measure and the number of viable pups as the covariate.

and +40%,  $P=0.047$ , respectively) (Figure 6.2c). IGF-I and Leu<sup>27</sup>-IGF-II increased total uptake of AIB into fetal tissues combined compared with vehicle (+33%,  $P = 0.045$ ; +30%,  $P = 0.05$ , respectively) (Figure 6.2c). Total AIB uptake by fetal muscle was 73% greater following maternal IGF-I treatment compared with IGF-II ( $P = 0.029$ ) (Figure 6.2c).

#### *6.2.1.6 Sustained effect of exogenous maternal IGF on the clearance of methyl glucose and amino isobutyric acid from the maternal circulation*

To determine whether early to mid pregnancy exogenous IGF treatment of the mother altered the clearance of MG or AIB from the maternal circulation in late pregnancy, following a bolus injection, maternal blood was sampled every 2 minutes for 20 minutes (Figure 6.3).

Maternal IGF-I treatment increased maternal plasma MG at 4 (+87%,  $P = 0.07$ ), 6 (+61%,  $P = 0.07$ ), 8 (+67%,  $P = 0.068$ ), 10 (+55%,  $P = 0.048$ ), 12 (+81%,  $P = 0.02$ ), 14 (+83%,  $P = 0.03$ ) and 18 (+63%,  $P = 0.07$ ) minutes following analogue administration, although some of these did not reach statistical significance (Figure 6.3a). Exogenous IGF-II or Leu<sup>27</sup>-IGF-II treatment in early to mid pregnancy did not alter the clearance of MG from maternal plasma in late pregnancy.

Mothers treated with Leu<sup>27</sup>-IGF-II, had greater plasma AIB concentrations at 2 (+292%,  $P = 0.048$ ) and subsequently at 20 (+257%,  $P = 0.022$ ) minutes following administration, compared with vehicle animals, with no effect between these time points. However, there was no effect of IGF-I or IGF-II treatment of the mother on AIB clearance from maternal plasma (Figure 6.3b).

#### *6.2.1.7 Sustained effect of exogenous maternal IGF on uptake of methyl glucose by tissues of the mother*

Maternal Leu<sup>27</sup>-IGF-II treatment in early to mid pregnancy increased total uptake of MG by maternal lung although this did not quite reach statistical significance (+33%,  $P = 0.066$ ). However, Leu<sup>27</sup>-IGF-II treatment reduced total MG uptake by the maternal retroperitoneal fat compared to vehicle (-58%,  $P = 0.004$ ), IGF-I (-69%,  $P = 0.038$ ) and IGF-II (-61%,  $P = 0.049$ ) treatment groups, near term (Table 6.4). There was no effect of maternal IGF treatment on MG uptake by individual maternal organs or tissues. Overall, the uptake of MG per gram of tissue was greatest in the maternal kidney and lowest in the interscapular fat (Table 6.4).

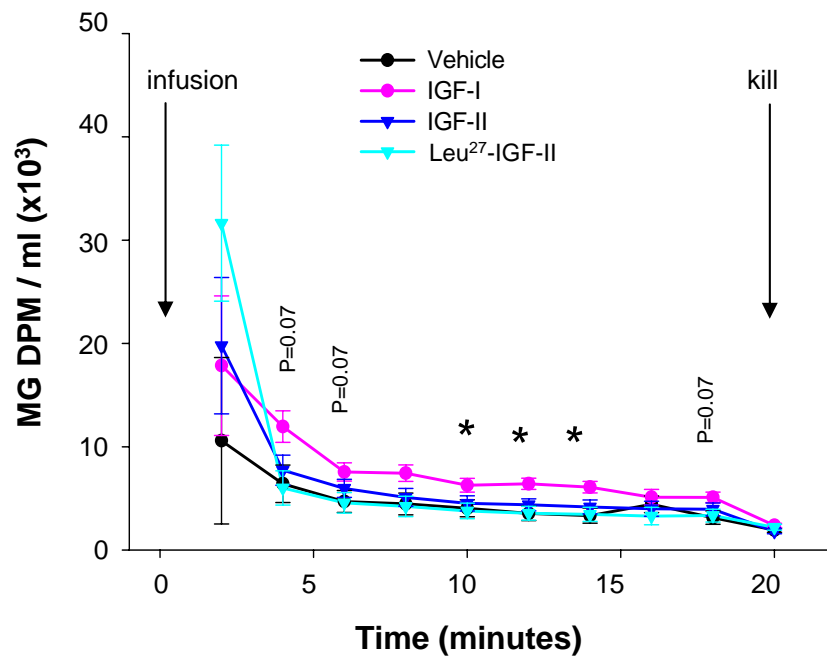


Figure 6.3 Sustained effect of maternal IGF treatment in early to mid pregnancy on the clearance of methyl-D-glucose from the maternal circulation. Maternal blood was sampled every 2 minutes for 20 minutes following bolus injection of [<sup>3</sup>H]-methyl-D-glucose (MG). Data are from 4-7 mothers per treatment and values are expressed as means DPM / ml  $\pm$  SEM. Asterisks denote statistically significant difference of IGF-I compared to the vehicle group,  $P < 0.048$ , with P values also displayed for time points where significance was almost reached. Data was analyzed using Repeated Measures ANOVA.

**Table 6.4 Sustained effect of maternal IGF treatment in early to mid pregnancy on maternal tissue methyl glucose uptake**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number of dams	6	7	6	4
Brain (DPM / gram)	189 ± 32	253 ± 29	195 ± 30	170 ± 35
(total DPM)	744 ± 139	1060 ± 125	794 ± 129	673 ± 152
Heart (DPM / gram)	133 ± 41	247 ± 34	201 ± 40	162 ± 48
(total DPM)	289 ± 85	524 ± 72	438 ± 85	382 ± 101
Liver (DPM / gram)	314 ± 74	414 ± 56	358 ± 59	387 ± 73
(total DPM)	8139 ± 1745	11739 ± 1313	9027 ± 1388	10061 ± 1642
Lungs (DPM / gram)	211 ± 35	270 ± 31	221 ± 30	215 ± 41
(total DPM)	896 ± 180 <sup>a</sup>	1075 ± 159 <sup>ac</sup>	1034 ± 156 <sup>ac</sup>	1188 ± 276 <sup>c</sup>
Kidneys (DPM / gram)	341 ± 142	702 ± 117	502 ± 122	466 ± 174
(total DPM)	1568 ± 727	3543 ± 598	2359 ± 623	2156 ± 721
Spleen (DPM / gram)	154 ± 36	222 ± 34	192 ± 37	174 ± 41
(total DPM)	175 ± 49	311 ± 46	307 ± 51	214 ± 55
Triceps (DPM / gram)	77 ± 35	148 ± 35	102 ± 32	93 ± 38
(total DPM)	171 ± 81	330 ± 80	227 ± 74	200 ± 85
Gastroc. (DPM / gram)	145 ± 43	241 ± 38	186 ± 40	108 ± 57
(total DPM)	271 ± 85	487 ± 77	364 ± 80	194 ± 93
Inters (DPM / gram)	43 ± 10	64 ± 9	36 ± 9	41 ± 12
(total DPM)	433 ± 120	573 ± 108	328 ± 112	295 ± 128
Periren (DPM / gram)	48 ± 28	107 ± 25	67 ± 26	96 ± 37
(total DPM)	220 ± 140	501 ± 126	276 ± 130	321 ± 154
Retro (DPM / gram)	72 ± 31	92 ± 25	64 ± 26	45 ± 22
(total DPM)	487 ± 236 <sup>a</sup>	658 ± 194 <sup>a</sup>	514 ± 202 <sup>a</sup>	204 ± 40 <sup>b</sup>

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Inters, interscapular fat; Periren, perirenal fat; Retro, retroperitoneal fat. Different superscripts denote difference between groups, a vs b, P < 0.05 (significant) or a vs c P = 0.066 (not significant), as determined by as determined by Univariate ANOVA with Bonferoni Post Hoc Tests.



Maternal IGF-I increased MG uptake by maternal muscle per gram by 78% (mean DPM / gram  $\pm$  SEM; vehicle:  $221 \pm 68$ ; IGF-I:  $394 \pm 61$ ; IGF-II:  $289 \pm 63$ ; Leu<sup>27</sup>-IGF-II  $186 \pm 74$ ,  $P = 0.058$ ) and visceral tissue by 97% (mean DPM / gram  $\pm$  SEM; vehicle:  $975 \pm 222$ ; IGF-I:  $1921 \pm 200$ ; IGF-II:  $1609 \pm 207$ ; Leu<sup>27</sup>-IGF-II:  $1078 \pm 240$ ,  $P = 0.014$ ) and combined internal organs and tissues by 101% (mean DPM / gram  $\pm$  SEM; vehicle:  $1454 \pm 351$ ; IGF-I:  $2935 \pm 339$ ; IGF-II:  $2371 \pm 332$ ; Leu<sup>27</sup>-IGF-II:  $1274 \pm 365$ ,  $P=0.007$ ).

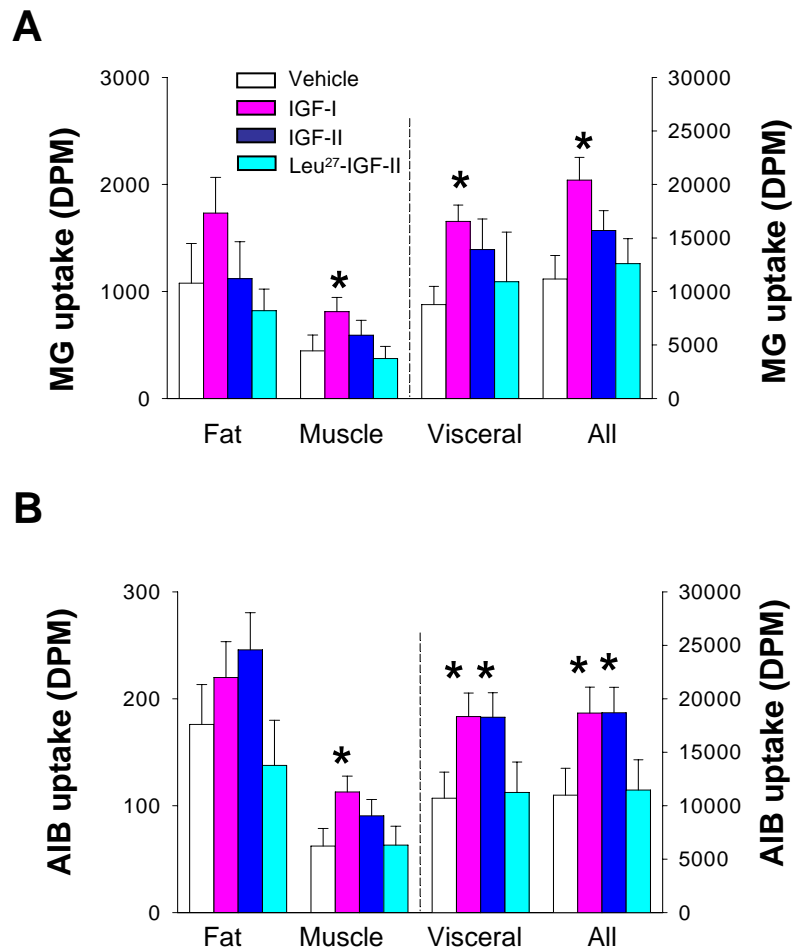
Exogenous maternal IGF-I increased the total MG uptake into maternal muscle (+82%,  $P = 0.046$ ), visceral (+89%,  $P = 0.016$ ) and all tissues combined (+102%,  $P = 0.019$ ) (Figure 6.4a).

*6.2.1.8 Sustained effect of exogenous maternal IGF on uptake of amino isobutyric acid by tissues of the mother*

Early to mid pregnancy maternal IGF-II treatment increased maternal spleen per gram and total AIB uptake near term, compared with vehicle treated dams (+98%,  $P = 0.039$ ; +310%,  $P = 0.002$ , respectively) (Table 6.5). There was a 2.3 and 2.4-fold difference in per gram and total uptake of AIB by the brain between IGF-II and Leu<sup>27</sup>-IGF-II treated mothers ( $P = 0.068$ ;  $P = 0.063$ , respectively) and a 4-fold difference in total retroperitoneal fat AIB uptake ( $P = 0.005$ ) (Table 6.5). Across all treatments, the uptake of AIB per gram of tissue was greatest in the maternal liver and lowest in the brain and interscapular fat of vehicle treated mothers and lowest in the brain in IGF and Leu<sup>27</sup>-IGF-II treated animals (Table 6.5).

Maternal IGF-I and IGF-II increased AIB uptake per gram by maternal visceral tissues by 54% and 83% compared with vehicle, respectively (mean DPM / gram  $\pm$  SEM; vehicle:  $670 \pm 91$ ; IGF-I:  $1031 \pm 81$ ; IGF-II:  $1225 \pm 84$ ; Leu<sup>27</sup>-IGF-II:  $857 \pm 107$ ,  $P = 0.014$  and  $P = 0.002$ , respectively) and summed internal organs and tissues by 52% and 80%, respectively (mean  $\pm$  SEM; vehicle:  $736 \pm 96$ ; IGF-I:  $1118 \pm 92$ ; IGF-II:  $1319 \pm 91$ ; Leu<sup>27</sup>-IGF-II:  $996 \pm 121$ ,  $P = 0.007$  and  $P = 0.002$ , respectively).

Maternal IGF-I increased total AIB uptake by maternal muscle (+82%,  $P = 0.044$ ) and both IGF-I and IGF-II treatment of the mother increased total AIB uptake by visceral tissues (both +71% and  $P = 0.048$ ) and combined internal organs and tissues (both +70% and  $P = 0.044$ ) (Figure 6.4b).



**Figure 6.4 Sustained effect of maternal IGF treatment in early to mid pregnancy on total maternal tissue substrate utilization.** Total maternal tissue uptake of [<sup>3</sup>H]-methyl-D-glucose (a) and [<sup>14</sup>C]-aminoisobutyric acid (b) were determined. Data are from 6 vehicle, 7 IGF-I, 6 IGF-II and 4 Leu<sup>27</sup>-IGF-II mothers. Values are expressed as mean total DPM ± SEM. Asterisks denote a statistically significant difference compared to the vehicle group,  $P < 0.048$ , as determined by a General Linear Univariate ANOVA with Bonferoni Post Hoc Tests.

**Table 6.5 Sustained effect of maternal IGF treatment in early to mid pregnancy on maternal tissue amino isobutyric acid uptake**

	Vehicle	IGF-I	IGF-II	Leu <sup>27</sup> -IGF-II
Number of dams	6	7	6	4
Brain (DPM / gram)	7.7 ± 1.3 <sup>ac</sup>	6.2 ± 1.2 <sup>ac</sup>	8.9 ± 1.2 <sup>a</sup>	3.9 ± 1.4 <sup>c</sup>
(total DPM)	31 ± 5.4 <sup>ac</sup>	26 ± 4.8 <sup>ac</sup>	36 ± 5.0 <sup>a</sup>	15 ± 1.4 <sup>c</sup>
Heart (DPM / gram)	38 ± 21	79 ± 17	91 ± 20	83 ± 25
(total DPM)	83 ± 44	171 ± 37	196 ± 44	196 ± 55
Liver (DPM / gram)	526 ± 138	570 ± 103	618 ± 109	386 ± 133
(total DPM)	13766 ± 3417	15871 ± 2570	15659 ± 2718	10020 ± 3331
Lungs (DPM / gram)	19 ± 11 <sup>ac</sup>	8.7 ± 9.3 <sup>a</sup>	9.7 ± 9.2 <sup>a</sup>	50 ± 12 <sup>c</sup>
(total DPM)	77 ± 23 <sup>a</sup>	36 ± 18 <sup>a</sup>	41 ± 18 <sup>a</sup>	274 ± 64 <sup>b</sup>
Kidneys (DPM / gram)	186 ± 33	220 ± 27	281 ± 28	245 ± 37
(total DPM)	846 ± 182	1108 ± 150	1329 ± 156	1134 ± 187
Spleen (DPM / gram)	94.9 ± 23 <sup>a</sup>	109 ± 21 <sup>ab</sup>	188 ± 23 <sup>b</sup>	107 ± 26 <sup>ab</sup>
(total DPM)	110 ± 63 <sup>a</sup>	153 ± 58 <sup>ab</sup>	341 ± 64 <sup>b</sup>	136 ± 68 <sup>ab</sup>
Triceps (DPM / gram)	19 ± 6	34 ± 6.2	31 ± 5.7	20 ± 6.7
(total DPM)	42 ± 15	77 ± 15	69 ± 14	44 ± 16
Gastroc. (DPM / gram)	12 ± 5.2	14 ± 4.6	11 ± 4.8	15 ± 6.9
(total DPM)	22 ± 11	32 ± 9.8	21 ± 10	27 ± 14
Inters (DPM / gram)	7.7 ± 2.1	8.9 ± 1.9	7.5 ± 1.9	5.2 ± 2.3
(total DPM)	78 ± 24	78 ± 21	72 ± 22	39 ± 25
Periren (DPM / gram)	8.2 ± 3.2	13 ± 2.9	13 ± 3.0	14 ± 4.1
(total DPM)	40 ± 16	61 ± 15	53 ± 15	62 ± 21
Retro (DPM / gram)	11 ± 2.8	12 ± 2.3	15 ± 2.4	7.0 ± 2.9
(total DPM)	74 ± 23 <sup>ab</sup>	81 ± 19 <sup>ab</sup>	120 ± 19 <sup>a</sup>	36 ± 18 <sup>b</sup>

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Inters, interscapular fat; Periren, perirenal fat; Retro, retroperitoneal fat. Different superscripts denote significant difference between groups, a vs b,  $P < 0.05$  (significant) and a vs c  $P = 0.068$  (not significant), as determined by Univariate ANOVA with Bonferoni Post Hoc Tests.

#### *6.2.1.9 Sustained exogenous maternal IGF on the concentration of metabolites in the fetal circulation*

Maternal IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II treatment increased fetal circulating amino acid concentrations (+196%, P=0.026; +137%, P=0.029; +339%, P = 0.008, respectively) and maternal IGF-I reduced fetal circulating cholesterol concentrations (-30%, P=0.049) compared to vehicle, near term (Figure 6.5a). There was no effect of treatment on fetal plasma glucose, triglyceride or free fatty acid concentrations (Figure 6.5a).

#### *6.2.1.10 Sustained effects of exogenous maternal IGF on maternal circulating metabolite concentrations*

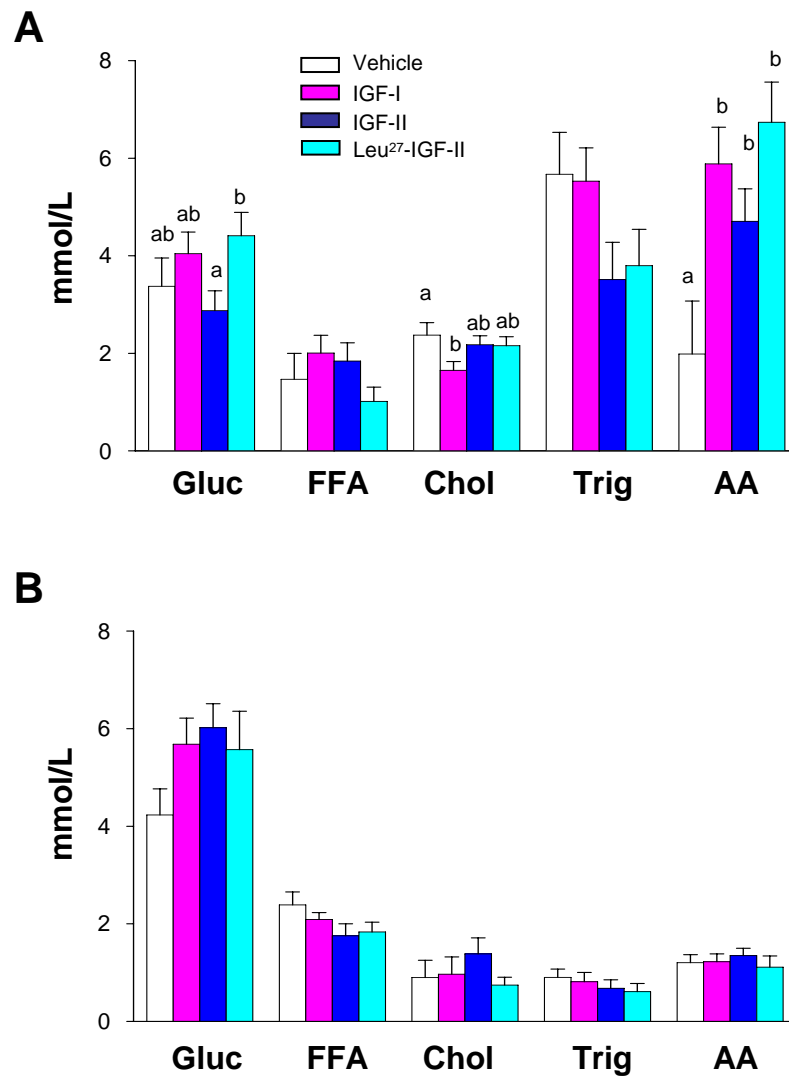
Maternal IGF treatment did not alter circulating concentrations of glucose, free fatty acids, amino acids, triglycerides or cholesterol in the mother near term (Figure 6.5b).

#### *6.2.1.11 Sustained effect of exogenous maternal IGF on fetal to maternal metabolite concentration gradients*

Maternal IGF-II treatment increased the ratio of maternal plasma glucose concentration to that in fetal plasma (estimated marginal mean ratio  $\pm$  SEM; vehicle:  $1.3 \pm 0.3$ ; IGF-I:  $1.4 \pm 0.3$ ; IGF-II:  $2.1 \pm 0.4$ ; Leu<sup>27</sup>-IGF-II:  $1.3 \pm 0.3$ , P = 0.035). Maternal IGF-I, IGF-II and Leu<sup>27</sup>-IGF-II reduced the ratio of maternal plasma amino acid concentration to that in fetal plasma (estimated marginal mean ratio  $\pm$  SEM; vehicle:  $0.61 \pm 0.05$ ; IGF-I:  $0.21 \pm 0.05$ ; IGF-II:  $0.29 \pm 0.05$ ; Leu<sup>27</sup>-IGF-II:  $0.16 \pm 0.06$ , effect of treatment P = 0.027, P = 0.05, P = 0.013, respectively). There was no significant effect of treatment on the concentration gradient of other metabolites between the fetal and maternal plasma (data not shown).

#### *6.2.1.12 Associations of MG uptake with placental morphology*

To determine the association of placental and fetal MG content with previously determined structural correlates of placental function near term (Chapter 5, p 139), correlations between these parameters were determined. Overall, MG uptake by placenta (DPM / gram) was correlated positively with the placental total surface area for exchange ( $r = 0.39$ , P = 0.039). Total placental MG uptake correlated positively with volume for placental exchange ( $r = 0.37$ , P = 0.019), the placental total surface area for exchange ( $r = 0.47$ , P = 0.001) and volume of maternal blood spaces within the labyrinth



**Figure 6.5** Sustained effect of maternal IGF treatment in early to mid pregnancy on circulating metabolites in the fetus (a) and mother (b). Fetal data are from all fetuses of all mothers (4-7 per treatment) and values are expressed as estimated marginal means adjusted for the number of viable pups  $\pm$  SEM. Maternal data are expressed as means  $\pm$  SEM. AA, amino acids; Chol, cholesterol; FFA, free fatty acids; Gluc, glucose; Trig, triglycerides. Different superscripts denote a statistically significant difference between treatments, a vs b  $P < 0.049$ . Fetal data were analysed by Linear Mixed Model Repeated Measures with Sidak Post Hoc test, using the mother as the subject, the fetus as the repeated measure and the number of viable pups per litter as the covariate. Maternal data were analysed by General Linear Univariate ANOVA with Bonferoni Post Hoc Tests

( $r = 0.32$ ,  $P = 0.035$ ). Overall, fetal plasma and tissue MG values were correlated positively with volume for placental exchange ( $r = 0.35$ ,  $P = 0.027$  and  $r = 0.38$ ,  $P = 0.025$ , respectively) and that of maternal blood spaces ( $r = 0.47$ ,  $P = 0.003$  and  $r = 0.40$ ,  $P = 0.023$ , respectively).

#### *6.2.1.13 Associations of MG uptake with maternal hormones*

Treating the mother with IGF during early to mid pregnancy altered the associations of placental and fetal MG content near term with previously determined maternal circulating estradiol concentrations (Chapters 4 and 5, p 122 and 134). However, maternal progesterone concentrations did not correlate with any parameter analysed (data not shown). There was no association of maternal hormones with AIB uptake or circulating metabolite concentrations in the fetus or mother (data not shown).

Maternal plasma estradiol concentration of vehicle treated animals correlated negatively with placental MG uptake and transfer capacity ( $r = -0.86$ ,  $P = 0.001$  and  $r = -0.85$ ,  $P = 0.002$ , respectively), whereas it was positively correlated in IGF-I ( $r = 0.56$ ,  $P = 0.011$  and  $r = 0.70$ ,  $P = 0.017$ , respectively) and IGF-II ( $r = 0.85$ ,  $P < 0.0001$  and  $r = 0.88$ ,  $P < 0.0001$ , respectively), but there was no association in Leu<sup>27</sup>-IGF-II treated mothers. In vehicle treated animals, maternal circulating estradiol was associated negatively with total uptake of MG by maternal tissues ( $r = -0.96$ ,  $P = 0.003$ ) but in IGF-I and Leu<sup>27</sup>-IGF-II treated mothers there was no correlation and following IGF-II treatment, these parameters were positively correlated ( $r = 0.84$ ,  $P = 0.036$ ).

### 6.3 Discussion

Here we demonstrate for the first time that treating the mother with either IGF during early to mid pregnancy has a sustained effect on placental transport of nutrients to the fetus and influences nutrient partitioning and likely metabolism, in the mother and conceptus near term. This extends, and provides new insight into, our previous observations that increasing the abundance of IGF-I and IGF-II in the maternal circulation during early to mid pregnancy enhanced fetal growth and survival near term (Chapter 5).

In this chapter, we have shown that early to mid pregnancy maternal IGF treatment, increased placental uptake and transfer of methyl glucose to the fetus in late pregnancy. As glucose is the primary source of energy for the fetus and the placenta (Owens 1991), enhanced placental transfer of glucose would directly increase fetal energy supplies to promote growth. This was certainly the case for maternal IGF-I treatment in the current study, and for both IGF treatments in our previous, larger study (Chapter 5), where fetal growth and survival were increased near term.

In mothers treated with IGF-II in particular, in early to mid pregnancy, enhanced placental transfer and uptake of methyl glucose by the fetus near term was demonstrated but may be secondary to sustained effects on placental structural development exerted primarily through IGF2R. IGF-II treatment in early to mid pregnancy increased the cross-sectional area, proportion and volume of the placental exchange region, as well as the total surface area of placenta functioning in exchange in late gestation (refer to Chapter 5, p 139). These structural changes were predicted to enhance the capacity of the placenta to deliver substrate and are consistent with the observed increase in methyl glucose transfer in late pregnancy, demonstrating here with both IGF-II and Leu<sup>27</sup>-IGF-II treatments. Indeed, we found positive correlations between a variety of placental structural parameters and placental methyl glucose transport to the fetus.

In contrast to IGF-II, maternal IGF-I treatment in early to mid pregnancy, did not alter the structure of the placental exchange region (Chapter 5). Circulating IGF-I in the mother may influence other determinants of placental transport capacity including the abundance, localisation and affinity of transporter proteins at the exchange interface, placental blood flow, concentration gradients of substrate between the mother and fetus, and placental consumption and modification of substrates. Indeed, maternal circulating glucose concentrations tended to be elevated by both IGFs and the concentration gradient

between the maternal and fetal circulation was increased in IGF-II treated animals. These alterations in maternal glucose concentration may have been partly responsible for increased placental methyl glucose transfer as net glucose transport to the fetus is dependent on maternal circulating glucose concentrations.

Uptake and transport of glucose across the placenta occurs via facilitated carrier-mediated diffusion (Schneider, Calderon *et al.* 1981) which, in the human, mouse, rat and sheep placenta, primarily involves the glucose transporter (GLUT) isoforms-1 and -3 (Currie, Bassett *et al.* 1996; Das, Sadiq *et al.* 1998; Ehrhardt and Bell 1997; Hahn, Hartmann *et al.* 1995; Illsley 2000; Jansson, Wennergren *et al.* 1993; Tadokoro, Yoshimoto *et al.* 1996; Takata, Kasahara *et al.* 1994; Wooding, Fowden *et al.* 2005; Zhou and Bondy 1993), although GLUT-4 and -12 placental expression have also been described (Ericsson, Hamark *et al.* 2005b; Gude, Stevenson *et al.* 2003). Glucose transporters are regulated by factors including glucose and glycolytic substrates (Klip, Tsakiridis *et al.* 1994; Sasson, Kaiser *et al.* 1997), oxygen (Ebert, Firth *et al.* 1995; Esterman, Greco *et al.* 1997; Hayashi, Sakata *et al.* 2004) and also by insulin, IGF-I and IGF-II (Ericsson, Hamark *et al.* 2005a; Maher and Harrison 1990; Wilson, Mitsumoto *et al.* 1995).

In a first trimester trophoblast cell line, exposure to either IGF increased glucose transporter activity (Gordon, Zimmerman *et al.* 1995; Kniss, Shubert *et al.* 1994) and in rats, maternal IGF-I treatment increased glucose transporter mRNA expression of *Slc2a1* and *Slc2a3*, which encode GLUT-1 and GLUT-3, respectively (Bauer, Harding *et al.* 1998). Furthermore, GLUT-1 expression on the basal membrane of the placental barrier is positively regulated by IGF-I (Baumann, Deborde *et al.* 2002). The latter observation is especially noteworthy, as GLUT-1 protein density on the basal membrane in the human placenta is thought to be the rate-limiting step in transplacental glucose transfer (Vardhana and Illsley 2002). This is consistent with reports of accelerated fetal growth in insulin-dependent diabetes mellitus being associated with increased GLUT-1 activity on the basal membrane (Gaither, Quraishi *et al.* 1999) and enhanced placental glucose transport capacity (Jansson, Ekstrand *et al.* 2001).

IGF-I may also have enhanced methyl glucose uptake and transfer to the fetus indirectly by increasing maternal blood flow to the placenta. During pregnancy, the maternal spiral arterioles that supply the uterus are transformed by invading placental trophoblasts into dilated vessels, unresponsive to maternal vasocontrol (Aplin 1991; Lyall 2005; Pijnenborg, Bland *et al.* 1983; Pijnenborg, Vercruysse *et al.* 2006). This normal physiological process permits uninterrupted high conductance flow of blood from the mother



that constitutes 30-40% of the total maternal cardiac output (Martin 1968). In culture, IGF-I stimulates trophoblast migration and invasion (Lacey, Haigh *et al.* 2002) through elevated matrix metalloproteinase-9 activity (Hills, Elder *et al.* 2004), activation of integrins (Kabir-Salmani, Shiokawa *et al.* 2004; Kabir-Salmani, Shiokawa *et al.* 2003) and altered cytoskeletal organisation (Kabir-Salmani, Shiokawa *et al.* 2002). It also inhibits vasoconstrictors, such as thromboxane B2 and prostaglandin F2  $\alpha$ , in human term placental explants (Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995). Placental synthesis of IGF-I is normally low (particularly compared to IGF-II) in most species analyzed, including the guinea pig and human (Han, Bassett *et al.* 1996; Han and Carter 2000; Han, Carter *et al.* 1999). However, in the IGF-I treated mother, elevated maternal circulating IGF-I concentrations may act in an endocrine fashion and influence the function of trophoblast surrounding or migrating within uterine spiral arteries. Therefore, elevated maternal circulating IGF-I may promote uterine vascular remodelling, which would permit increased perfusion of the placenta and indirectly increased substrate flux to the fetus.

Despite effects of exogenous maternal IGFs on placental transport of methyl glucose, there was no effect of IGF-I or IGF-II treatment on placental uptake and transfer of the amino isobutyric acid in late gestation. This was unexpected, as in the current study fetuses from IGF treated mothers had substantially increased plasma  $\alpha$ -amino nitrogen concentrations near term. These outcomes suggest that maternal IGF treatment may not alter the sodium-dependent, System A transporters, which are responsible for amino isobutyric acid and neutral amino acid (alanine, serine, proline and glutamine) transfer, but rather may target other amino acid transporter systems. There are at least 15 different amino acid transporter systems identified in the human and rat placenta (Jansson 2001; Regnault, Friedman *et al.* 2005), which display overlapping substrate specificity (Battaglia 2002). It is also important to remember that in some species like the guinea pig, which possess a separate yolk sac (vitelline) placenta, substrates including amino acids may be transferred to the fetus via this route (Butt and Wilson 1968). Whether IGF treatment to the mother affected yolk sac transport has yet to be elucidated. Determining the fetal circulating amino acid profile in response to earlier maternal IGF treatment would provide more insight into which amino acid transporters may be affected in the placenta, providing a direct link with the observed increased fetal growth (Chapter 5). Indeed, in transgenic mice, elevated abundance of IGF-I due to growth hormone over-expression, or deficiency in IGF-II by gene ablation, altered placental expression of X-AG transporters which are responsible for sodium-dependent uptake and transfer of anionic amino acids like glutamate and aspartate (Matthews, Beveridge *et al.* 1999).

In direct contrast to findings with IGF supplementation, Leu<sup>27</sup>-IGF-II treatment, substantially increased placental System A amino acid uptake. As system A transports nonessential amino acids which are subjected to catabolism in the placenta, it is possible that Leu<sup>27</sup>-IGF-II treatment increased placental metabolism. This may have occurred indirectly as Leu<sup>27</sup>-IGF-II is an analogue of IGF-II, which selectively interacts with IGF2R due to reduced binding capacity for the IGF1R and InsR (80 and 220-fold lower affinity, respectively) (Sakano, Enjoh *et al.* 1991) and therefore would have reduced the availability of IGF2R for endogenous maternal IGF-II. This would permit endogenous IGF-II to interact with other receptors, including the InsR, which is known to mediate metabolic effects. Nevertheless, enhanced placental metabolism would be expected to increase placental function, which is consistent with increased methyl glucose and amino isobutyric acid transfer and improved pregnancy outcome in Leu<sup>27</sup>-IGF-II treated pregnancies. Certainly, determining the maternal plasma concentration of IGF-II in the mothers treated with Leu<sup>27</sup>-IGF-II may provide some insight.

Surprisingly, fetal cholesterol concentrations near term were reduced by earlier maternal IGF-I supplementation. The liver is the main source of cholesterol in the guinea pig fetus (Yount and McNamara 1991). As previous investigative analysis determined that the fractional weight of fetal liver was reduced in response to maternal IGF-I supplementation (Chapter 5, p 146), we speculate that a reduction in fetal plasma cholesterol is a direct consequence of inadequate liver growth, proportionate to total fetal weight.

Both maternal IGF treatments increased methyl glucose fetal tissue utilization while IGF-I also increased that of amino isobutyric in the fetal visceral organs, near term. Furthermore, there was increased utilization of methyl glucose and amino isobutyric acid per gram of several fetal tissues of IGF treated mothers, without an alteration in wet weight (Chapter 5, p 146). We therefore speculate, that in the larger fetuses of the IGF treated mothers particular fetal tissues may be functionally more active and therefore metabolising substrate more efficiently, contributing to the rapid growth of the fetus in late gestation. This altered fetal growth trajectory may have been established during treatment and sustained until term, as fetuses of IGF-I treated mothers on day 35 (Chapter 3, p 112), or treated with either IGF on day 40 (Sohlstrom, Fernberg *et al.* 2001) were also heavier than controls.

Interestingly, in the current study, early pregnancy treatment of the guinea pig with either IGF also increased uptake of methyl glucose and amino isobutyric acid by maternal tissues in late pregnancy. Particularly in the case of IGF-II, this effect appeared to be independent of the IGF2R, as there was no impact of Leu<sup>27</sup>-IGF-II treatment on maternal substrate utilization. This suggested that either maternal

tissues have reduced abundance of IGF2R, or much more likely, that IGF2R does not mediate metabolic effects in cells. Nevertheless, despite increased maternal substrate utilization, maternal organ weights were not increased and fetal and placental growth were not compromised near term in response to earlier maternal IGF treatment (Chapter 5). These suggest that the IGF treated mothers may be metabolically more active than vehicle-treated controls. In humans, total energy expenditure increases by 19% by the third trimester of pregnancy compared with non-pregnant women (Butte 2005) and during normal pregnancy, endogenous hepatic glucose production (Assel, Rossi *et al.* 1993; Catalano and Hollenbeck 1992; Catalano, Roman-Drago *et al.* 1998; Kalhan, D'Angelo *et al.* 1979), the 24-h respiratory quotient and basal and sleeping metabolic rates all increase (Bronstein, Mak *et al.* 1995; Butte, Hopkinson *et al.* 1999; Denne, Patel *et al.* 1991; van Raaij, Schonk *et al.* 1989). These maternal physiological changes presumably contribute to maternal adaptation to pregnancy to meet the increasing demands of the placenta and fetus.

Increased maternal amino isobutyric acid and methyl glucose utilization in late pregnancy in response to IGF treatment may reflect a maternal metabolic strategy that enabled maternal organs to increase their functional capacity and energy expenditure. Indeed, we have found in our guinea pig cohorts that total conceptus mass may be equal to as much as 50% of maternal weight near term, which is likely to place huge metabolic demands on the mother. Certainly, maternal plasma glucose concentrations tended to be increased in response to earlier maternal IGF treatment, suggestive of increased maternal hepatic glucose production and improved maternal metabolism. Furthermore, increased concentration of glucose in the maternal circulation may have been a mechanism for increasing substrate availability for fetal growth. Indeed, in order to divert nutrients to the conceptus, the mother acquires a state of relative insulin-resistance as pregnancy advances (Catalano, Tyzbir *et al.* 1991; Catalano, Tyzbir *et al.* 1993; Catalano, Tyzbir *et al.* 1992; Ryan, O'Sullivan *et al.* 1985). Interestingly, in the current study, mothers treated with IGF-I during early to mid pregnancy displayed reduced clearance of methyl glucose from maternal plasma, although fetal, placental and maternal substrate uptakes were improved, near term. This may have reflected substantial differences in the response of other maternal and fetal tissues, not analysed in the current study, to maternal IGF treatment. Such tissues include other fat depots and skeletal muscles, the gastrointestinal tract, uterus and skin, which are known to vary in their endocrine characteristics, including insulin and related molecule sensitivities. In any case, it appears that IGFs in the mother may have promoted maternal adaptation to pregnancy, such as development of insulin resistance and altered maternal metabolism, thereby enhancing fetal growth and survival.

In conclusion, we have shown that increased maternal circulating IGF concentrations in early pregnancy sustainably increase maternal substrate utilization, placental MG transfer to the fetus and fetal utilization of MG and AIB, enhancing fetal growth and survival near term. Whether the latter are indirect consequences of enhanced maternal adaptation to pregnancy or whether maternal substrate utilization was increased in response to enhanced conceptus growth to meet the challenge of pregnancy, remain to be elucidated. Nevertheless, we speculate that effects of maternal IGF-II treatment on placental MG uptake and transfer are secondary to impacts on placental differentiation, which is primarily exerted via IGF2R (Chapter 5). In contrast, actions of exogenous IGF-I on placental transfer and fetal growth and substrate utilization are believed to be mediated through IGF1R, as it has little or no affinity for InsR and IGF2R, through influencing maternal tissues and possibly placental parameters not assessed in the present study. Overall, this study has provided further evidence for a major role of maternal IGFs in the regulation of nutrient partitioning during pregnancy. As insufficient supply of nutrients to the fetus is associated with IUGR (Cetin, Corbetta *et al.* 1990; Cetin, Radaelli *et al.* 2001; Economides and Nicolaides 1989), therapeutic approaches that increase maternal IGFs in early to mid pregnancy may be effective in ameliorating or preventing its development in pregnancies at risk. The challenge is to identify those women at risk of IUGR early in pregnancy before the fetus is compromised.

*Chapter 7 : Thesis discussion  
and conclusion*

## 7.1 Discussion

It is well established that, in humans onset of maternal blood flow to the placenta commences around 10 weeks of gestation and, prior to this time, the fetus and placenta are developing in a hypoxic environment which is essential for a healthy pregnancy outcome. The effects of hypoxia on placental trophoblast function are likely to be mediated locally through the regulation of growth factors. For instance, components of the IGF system are expressed at the feto-maternal interface, with IGF-II in particular, highly expressed by invading EVT's. Certainly, maternally-derived IGFs may also regulate fetal growth during pregnancy through actions on the placenta but the mechanisms involved have yet to be fully elucidated.

The studies described in this thesis indicate that oxygen and IGF-II interact at the feto-maternal interface and regulate placental invasion and growth. In addition, it has been demonstrated that maternal circulating IGFs in early to mid pregnancy regulate fetal growth and viability in late pregnancy, through enhancing placental substrate transfer capacity and influencing nutrient allocation between the mother and conceptus.

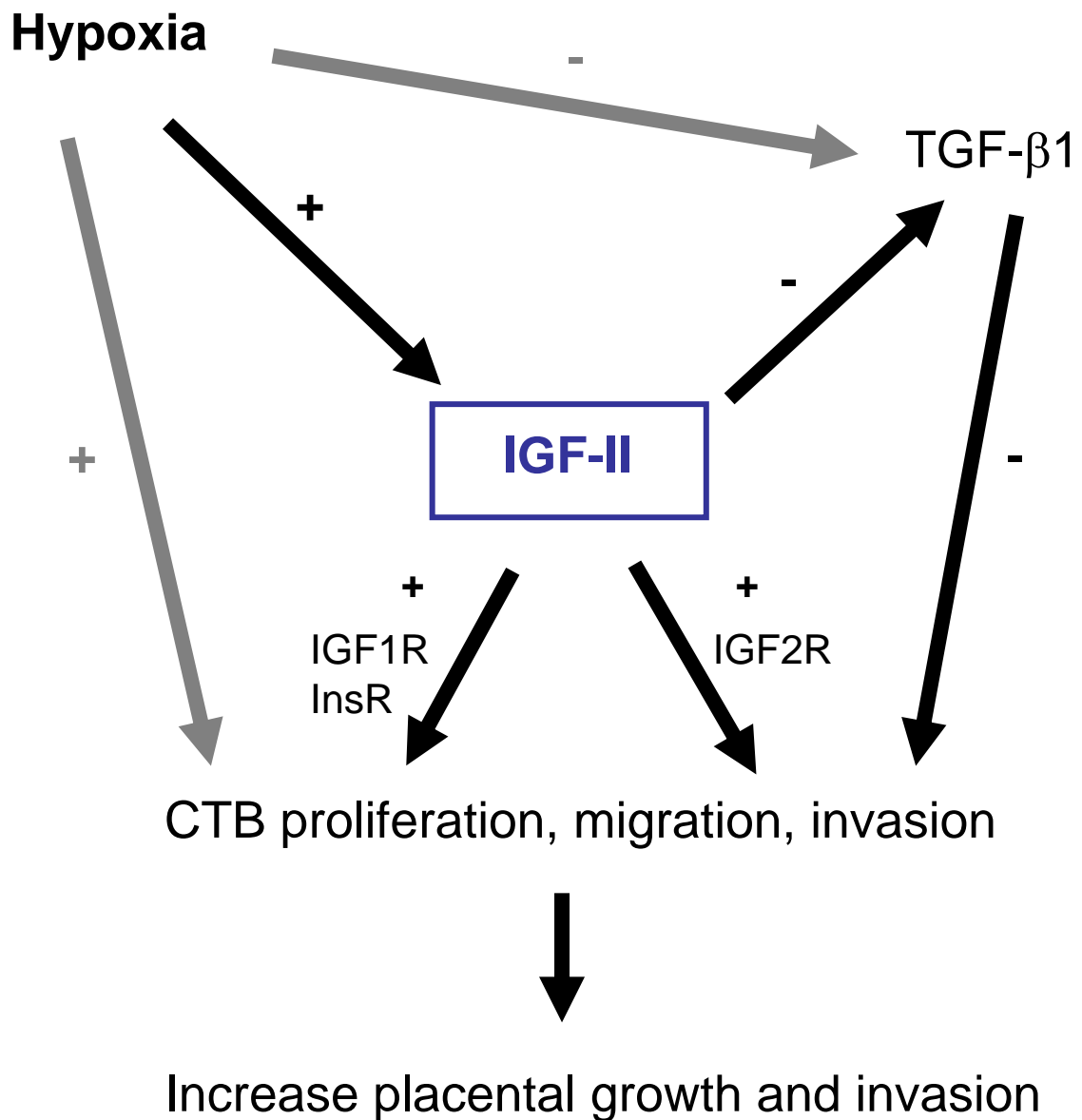
### 7.1.1 *Interaction of oxygen and IGF-II during early pregnancy in vitro*

In Chapter 3, first trimester human placental villous explants were prepared from pregnancies terminated between 7 and 12 weeks. Initial studies comparing the growth response of villous explants to culture under low oxygen atmospheres across the gestational ages sampled, revealed a change at 10 weeks of gestation. This was seen by declining villous outgrowth. Work of others has demonstrated that onset of maternal blood flow to the placenta occurs gradually from 10 weeks of gestation *in vivo* (Burton, Jauniaux *et al.* 1999; Rodesch, Simon *et al.* 1992). Thus, our findings showing reduced outgrowth of villous explants of greater than 10 weeks of gestation to low concentrations of oxygen are consistent with a response to hypoxic stress, as the placenta would have been exposed to higher oxygen concentrations *in vivo*. Furthermore, we detected an inverse relationship between villous outgrowth and gestational age of the placenta, suggesting that trophoblasts are programmed to proliferate and invade optimally in early stages of gestation, particularly at 7 to 8 weeks of gestation, when maternal blood perfusion of the placenta is minimal. Further experiments revealed that villous explants from placenta obtained at 7 or 8 weeks of gestation exposed to low oxygen atmospheres displayed enhanced villous tip outgrowth, increased *Igf2* gene expression and produced almost no active TGF- $\beta$ 1. Induction of *Igf2* transcription by hypoxia has been previously shown in other cell types

(Feldser, Agani *et al.* 1999; Kim, Bae *et al.* 1998; Steinbrech, Mehrara *et al.* 2000; Tucci, Nygard *et al.* 1998) and is believed to be mediated by HIF.

In our studies, we found no effect of oxygen availability on *Tgf $\beta$ 1* gene expression or total protein secretion, indicating that regulation of TGF- $\beta$ 1 by oxygen occurs at the protein activation level. Further experiments performed suggested that IGF-II may be mediating the observed effects of low oxygen on the placenta (Figure 7.1). In the present thesis, exogenous IGF-II stimulated villous explant outgrowth and inhibited TGF- $\beta$ 1 activation in villous explants cultured in high oxygen, mimicking the response of explants to low oxygen. Indeed, IGF-II stimulates trophoblast proliferation (Ohlsson, Holmgren *et al.* 1989), migration (Irving and Lala 1995; McKinnon, Chakraborty *et al.* 2000; McKinnon, Chakraborty *et al.* 2001) and invasion (Hamilton, Lysiak *et al.* 1998) in first trimester cell lines, *in vitro*, the former mediated via IGF1R (Ohlsson, Holmgren *et al.* 1989) or potentially via InsR (Morrione, Valentinis *et al.* 1997). Furthermore, IGF-II may have promoted villous outgrowth indirectly, by reducing activation of TGF- $\beta$ 1, which is well known for its inhibitory effects on trophoblast proliferation (Graham and Lala 1992), migration (Irving and Lala 1995) and invasion (Graham and Lala 1991; Graham and Lala 1992) *in vitro*.

Indeed, there is considerable overlap between the IGF and TGF- $\beta$  systems, as outlined in Chapter 1. Most noteworthy is IGF2R, which can bind both IGF-II and latent TGF- $\beta$ 1. The latter interaction results in activation of the TGF- $\beta$ 1 protein through a complex formed between IGF2R, uPA, uPAR and plasminogen (Godar, Horejsi *et al.* 1999; Leksa, Godar *et al.* 2005) (refer to Figure 1.14). As IGF2R cannot bind IGF-II and TGF- $\beta$ 1 simultaneously and the former has a greater affinity for IGF2R, increased expression of *Igf2* by exposure to hypoxia or exogenous IGF-II treatment of villous explants, is likely to reduce the availability of IGF2R for binding by latent TGF- $\beta$ 1 and thereby prevent its activation. Analysis of growth and TGF- $\beta$ 1 activation in villous explants exposed to low oxygen atmospheres in the presence of anti-sense RNA, small-interfering RNA or antibodies specific for IGF-II, would provide further evidence for the role of IGF-II in the effect of hypoxia on placental outgrowth. As *Igf1r*, *Igf2r* and *Upar* genes were unaltered by oxygen concentration or IGF-II, the effect of inhibitors of components of the IGF2R-uPAR complex and use of Leu<sup>27</sup>-IGF-II, an analogue selective for IGF2R, but not IGF1R or InsR, on TGF- $\beta$ 1 and trophoblast invasion, would complement the current findings. Furthermore, utilizing primary cultures of first trimester trophoblast, rather than villous explants, in these experiments would enable more quantitative measures of outgrowth by performing migration and invasion assays.



**Figure 7.1** IGF-II mediates the effect of hypoxia to promote human placental outgrowth in early pregnancy. At 7 to 8 weeks of gestation, prior to the onset of maternal blood flow to the placenta, hypoxia stimulates placental outgrowth, induces *Igf2* gene expression and inhibits TGF- $\beta$ 1 activation. Exogenous IGF-II mimics the effect of hypoxia in placenta exposed to high oxygen, as it enhances placental outgrowth and inhibits activation of TGF- $\beta$ 1.



Interestingly, TGF- $\beta$ 1 is involved in trophoblast terminal differentiation into syncytium, a process critical for functional maturation of the placenta in later gestation. Therefore, in later pregnancy, following maternal blood perfusion of the placenta and when IGF-II abundance is known to decline (Han, Bassett *et al.* 1996), we suspect there would be increased availability of IGF2R for interaction with and activation of, latent TGF- $\beta$ 1, which would transform placental functional development. From these studies we propose that, oxygen is the regulatory switch during pregnancy, influencing trophoblast proliferation and migration/invasion as opposed to trophoblast terminal differentiation through effects on IGF-II expression and TGF- $\beta$ 1 activation (Figure 7.2). Certainly, premature onset of maternal blood flow to the placenta is associated with early pregnancy loss, highlighting the significance of hypoxia in early gestation to pregnancy outcome (Hustin, Jauniaux *et al.* 1990; Jauniaux, Greenwold *et al.* 2003; Jauniaux, Zaidi *et al.* 1994). Importantly, regardless of the gestational age of the placenta, exogenous IGF-II enhanced villous explant outgrowth *in vitro*. Enhanced placental growth is expected to increase its functional capacity and thereby improve pregnancy outcome.

### 7.1.2 *Role of maternal IGFs during pregnancy in vivo*

Studies performed on genetically modified mice, have demonstrated the role of IGF-II, but not IGF-I, in placental development and function, whilst both IGFs are important for optimal fetal growth (Baker, Hardy *et al.* 1996; Constancia, Hemberger *et al.* 2002; DeChiara, Efstratiadis *et al.* 1990; Ferguson-Smith, Cattanaach *et al.* 1991; Liu, Baker *et al.* 1993; Louvi, Accili *et al.* 1997). This work implied that there may be distinct mechanisms employed by each IGF, for the regulation of fetal growth. Thus, the rest of the studies detailed in this thesis aimed to elucidate the roles and effects of IGF-I and IGF-II treatment to the mother during early to mid pregnancy (days 20 to 38 of pregnancy) on placental growth and function and pregnancy outcome *in vivo*. These studies were performed in pregnant female guinea pigs, which display a similar IGF axis to humans. In mid pregnancy, this treatment elevated the concentrations of IGF-I and IGF-II in the maternal plasma by 350% and 240%, respectively. Experiments in Chapter 4 sought to determine the acute effects of elevated maternal IGF circulating concentrations on placental function, maternal substrate utilization and fetal growth at day 35 of gestation. Studies described in Chapters 5 and 6 ascertained the sustained impacts of earlier IGF treatment to the mother on maternal and fetal body composition, placental development and function and nutrient partitioning near term, on day 62 of pregnancy.

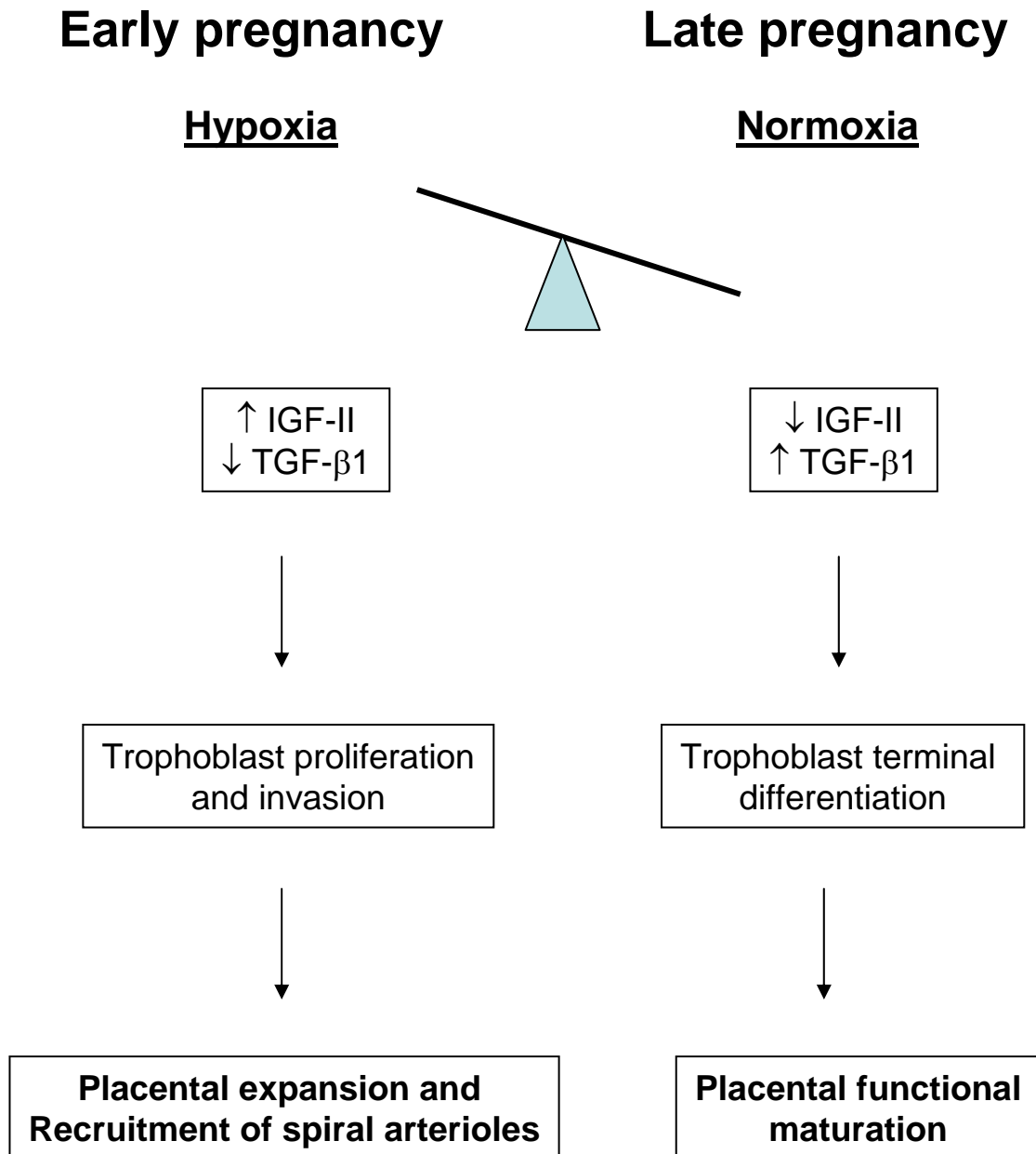


Figure 7.2 Proposed model of oxygen acting as the regulatory switch in placental development. In early pregnancy, when there is little to no blood flow to the placenta, IGF-II is abundant preventing activation of TGF-β1, resulting in direct and indirect stimulation of trophoblast proliferation and invasion. This promotes placental expansion and subsequent maternal blood flow to the placenta. In later gestation, when the placenta is perfused with maternal blood, oxygen concentrations rise and there is less IGF-II synthesis, there is abundant active TGF-β1, which induces trophoblast terminal differentiation by inhibiting proliferation and invasion. This results in functional maturation of the placenta.

### 7.1.2.1 IGF effects on the fetus

Both IGF treatments increased fetal survival and growth in late pregnancy, the latter of which was evident in mid pregnancy, in IGF-I treated mothers. This is consistent with a previous, similar study also undertaken in guinea pigs, demonstrating increased fetal growth on day 40 of pregnancy in response to earlier maternal IGF treatment (Sohlstrom, Fernberg *et al.* 2001) and the well acknowledged influence of IGFs on fetal growth in numerous species (Fowden 2003; Murphy, Smith *et al.* 2006; Owens 1991; Reik, Constancia *et al.* 2003; van Kleffens, Groffen *et al.* 1998). Analysis of fetal body composition revealed that the increase in fetal weight in late gestation in response to earlier maternal IGF supplementation was principally due to increased muscle mass and possibly enhanced bone growth (indicated by increased carcass weights). IGF-I treatment of the mother, however also reduced the fractional weight of the brain, spleen and liver in late gestation. Analysis of circulating metabolites revealed that fetuses of IGF-I treated mothers displayed reduced plasma cholesterol concentrations. The impact of these fetal alterations on postnatal health outcomes, have yet to be determined. Certainly, there is substantial evidence from human epidemiological and animal studies, including the guinea pig, demonstrating that alterations in fetal developmental can have impacts on the health of offspring in adult life, including increased risks of diabetes, heart disease and obesity (Barker 1998; Barker 2004a; Barker 2004b; Barker 2004c; Kind, Clifton *et al.* 2003; Kind, Clifton *et al.* 1999; Kind, Simonetta *et al.* 2002). Postnatal studies investigating metabolic profiles, blood pressure and immune and cognitive function in offspring of IGF treated mothers would better elucidate long-term outcomes. Nevertheless, we predict increased fetal muscle mass induced by earlier maternal IGF treatment, may prove to be metabolically beneficial in later life. Certainly, fetuses of IGF treated mothers appear to have an increased metabolic rate as fetal substrate utilization is increased near term.

### 7.1.2.2 IGF effects on the placenta

As maternal IGFs cannot be transported across the placenta (Brown and Thorburn 1989), we then determined whether alterations in fetal growth were secondary to effects on the placenta. In mid gestation, IGF-I, but not IGF-II increased placental weight. This was not sustained until term. Importantly, however, analysis of placental structural development in late gestation revealed that, IGF-II, but not IGF-I, enhanced the development of the labyrinth, the region dedicated to substrate exchange between the maternal and fetal circulations. Maternal IGF-II supplementation increased the area, proportion and volume of placenta devoted to exchange. More detailed inspection of the labyrinth revealed that the volumes of trophoblast and maternal blood spaces and total surface area of placenta participating in exchange were increased by earlier maternal IGF-II supplementation. These placental changes are the converse of those observed in undernourished guinea pigs which possess reduced

circulating IGF-II (Roberts, Sohlstrom *et al.* 2001a; Roberts, Sohlstrom *et al.* 2001b) or studies in mice that display perturbed placental *Igf2* expression (Constancia, Hemberger *et al.* 2002; Sibley, Coan *et al.* 2004). Furthermore, in the current studies, maternal IGF-II treatment reduced the proportion of placenta composed of interlobium (germinative region). Although these effects were not evident in mid gestation (Standen *et al.*, in preparation), such alterations near term reflect advanced placental maturation, as it is well established that the labyrinth expands at the expense of the interlobium in the final phase of guinea pig placentation (Kaufmann and Davidoff 1977; Roberts, Sohlstrom *et al.* 2001a). This increases placental transport capacity and efficiency in late gestation, when the substrate demand of the fetus is high and increasing.

To determine whether maternal IGF treatment had altered placental transport, we measured transfer of radiolabelled, non-metabolisable analogues of glucose and System A amino acids to the fetus. In accordance with anabolic effects of IGF-I treatment on the fetus and placenta in mid gestation and both IGF treatments on fetal growth in late pregnancy, placental uptake and transport of substrate to the fetus and fetal utilization, were enhanced. Maternal IGF-I administration, enhanced mid gestational placenta uptake and transfer of glucose and System A amino acid to the fetus, with both IGF treatments sustainedly enhancing placental uptake and transport of glucose to the fetus, in late gestation. Our findings are in agreement with *in vitro* experiments showing enhanced glucose and amino acid uptake by human trophoblasts (Bloxam, Bax *et al.* 1994; Karl 1995; Kniss, Shubert *et al.* 1994; Yu, Iwashita *et al.* 1998) and villous explants (Ericsson, Hamark *et al.* 2005a) in response to IGF stimulation *in vitro*. Interestingly, the activity of the placental System A transporters, responsible for placental delivery of small neutral amino acids to the fetus, was unaltered in late gestation by earlier IGF supplementation. This was interesting as fetuses of IGF supplemented mothers contained significantly elevated fetal plasma  $\alpha$ -amino nitrogen concentrations, an indication of total amino acid content. These suggest that other amino acid transporter systems are altered by earlier maternal IGF treatment. This finding is not surprising as studies performed in genetically modified mice have implicated IGFs in the regulation of placental cationic amino acid transporter systems (Matthews, Beveridge *et al.* 1999). Additionally, elevated maternal IGFs may promote yolk sac delivery of amino acids, as in the guinea pig, nutrients may be transferred to the fetus via this route throughout gestation (Butt and Wilson 1968). Certainly, defining the fetal plasma amino acid composition by HPLC or mass spectrometry and then determining the abundance of substrate transporters in the placenta and yolk sac in response to maternal IGFs would provide clarification.

Although maternal IGF-I treatment did not affect placental development in late gestation, it increased the ratio of fetal weight to placental weight, suggesting increased placental efficiency. Elevated maternal circulating IGF-I may have altered other determinants of placental function that were not measured in these studies. For instance, exogenous IGF-I has the potential to influence placental transporter expression (Bilan, Mitsumoto *et al.* 1992a; Bilan, Mitsumoto *et al.* 1992b; Fladeby, Skar *et al.* 2003; Phillips, Ferraz *et al.* 2005; Wilson, Mitsumoto *et al.* 1995), blood flow (Bilan, Mitsumoto *et al.* 1992a; Bilan, Mitsumoto *et al.* 1992b; Fladeby, Skar *et al.* 2003; Phillips, Ferraz *et al.* 2005; Schini-Kerth 1999; Siler-Khodr and Forman 1993; Siler-Khodr, Forman *et al.* 1995; Wilson, Mitsumoto *et al.* 1995; Yang, Chao *et al.* 2006) and trophoblast migration (Hills, Elder *et al.* 2004; Kabir-Salmani, Shiokawa *et al.* 2002; Kabir-Salmani, Shiokawa *et al.* 2004; Kabir-Salmani, Shiokawa *et al.* 2003; Lacey, Haigh *et al.* 2002), which affect placental substrate transfer. An examination of the effect of maternal IGF supplementation on these parameters would better clarify these speculations.

Despite differences in IGF effects on placental development, both IGF-I and IGF-II appeared to improve placental hormone synthesis, as indicated by elevated maternal plasma estradiol and progesterone concentrations in mid and/or late pregnancy. These were associated with fetal growth and placental development and have established roles in maintenance of pregnancy through modulating maternal physiology and adaptation to pregnancy (Butte 2000; Catalano 1999; Ryan and Enns 1988).

### *7.1.2.3 IGF effects on the mother*

Analysis of maternal body composition and substrate utilization suggested that maternal IGF treatment may have increased substrate transfer to the fetus during pregnancy by affecting the mother. There was no effect of elevated maternal circulating IGF-I or IGF-II on weight gain during or following cessation of treatment until near term. In mid pregnancy, at the time of treatment, we found an increase in the weight of maternal kidneys and spleen with IGF-I administration and a trend for increased heart mass with IGF-II. These effects were not sustained until late pregnancy, however, at this time, maternal adipose stores were depleted by earlier IGF-I treatment. These, mid and late gestational effects observed in the mother in response to IGF supplementation, suggest enhanced maternal adaptation during pregnancy. For instance, increased kidney, spleen and heart mass would be expected to promote maternal blood filtration and flow to the uterus. Furthermore, mobilisation of maternal fat depots would increase substrate availability in the maternal circulation, for transfer to the fetus.

Further studies measuring maternal substrate utilization, provided support for IGFs in the mother promoting maternal adaptation throughout pregnancy. Maternal IGF-I increased maternal muscle glucose and System A amino acid utilization in mid pregnancy. In later pregnancy, IGF-I increased glucose and System A amino acid utilization by muscle, visceral tissues and all tissues combined. IGF-II increased late gestational visceral organ and total tissue System A amino acid utilization. Despite increased maternal substrate utilization with increased IGF exposure of the mother during early to mid pregnancy, this did not significantly alter maternal growth and, importantly, increased placental delivery of nutrients to the fetus, enhancing fetal growth near term. These observations suggest that IGF supplementation altered maternal physiology, increasing the function and metabolism of maternal organs which, in turn, improved maternal and fetal health and conceptus growth during pregnancy. This speculation is supported by studies performed in women demonstrating that during pregnancy, their daily rate of carbohydrate oxidation increases to meet the demands of the conceptus (Butte 2000; Catalano 1999).

There was no effect of IGF supplementation on maternal plasma cholesterol,  $\alpha$ -amino nitrogen, free fatty acids and triglycerides in mid or late pregnancy. However, there was a trend for increased circulating glucose concentration in late pregnancy in mothers treated with either IGF. Women increase their hepatic glucose production during normal pregnancy (Assel, Rossi *et al.* 1993; Catalano, Roman-Drago *et al.* 1998; Catalano, Tyzbit *et al.* 1992), suggesting that this adaptive response to the pregnant state is accentuated in guinea pig mothers that were treated during early to mid pregnancy with IGF. We suspect that this may reflect a state of enhanced insulin resistance in the IGF treated mothers. Additionally, in mothers treated with IGF-I, there was a delay in the clearance of methyl glucose from the circulation, which indicating altered maternal insulin sensitivity. Analysis of maternal metabolic parameters during pregnancy in response to IGF administration would provide further information on these issues. Moreover, the consequence of reduced maternal adiposity on lactation in IGF-I treated dams is yet to be determined.

#### *7.1.2.4 Mechanism for distinct IGF actions*

Despite somewhat similar outcomes on fetal growth and survival, placental function and maternal substrate utilization, there were distinct effects of IGF treatment (Table 7.1). For instance, IGF-I infusion altered maternal body composition and did not affect placental structural development, while IGF-II altered placental development but not maternal body composition. We propose that, maternal IGFs may play complementary, but overlapping roles during pregnancy to ensure fetal growth and survival. IGF-I appears to predominantly influence maternal metabolism and fuel reserves, potentially increasing

Table 7.1 Summary of exogenous maternal IGF effects on the fetus, placenta and mother

Day 35	IGF-I	IGF-II
<b>Fetus</b>		
Litter	↔	↔
Weight	↑	↔
Substrate utilization	↑ MG ↑ AIB	↔
<b>Placenta</b>		
Weight	↑	↔
Structure**	↑ total area ↓ area for exchange	↔
Hormone synthesis	↔	↑ estradiol ↑ progesterone
Substrate uptake	↑ MG ↑ AIB	↔
<b>Mother</b>		
Weight gain	↔	↔
Body composition	↑ kidney mass ↑ spleen mass	↑ heart mass
Circulating metabolites	↔	↔
Components of IGF system in the circulation	↓ IGF-II ↑ IGFBP	↔
Substrate utilization	↑ MG ↑ AIB	↔
Day 62	IGF-I	IGF-II
<b>Fetus</b>		
Litter	↑ viability	↑ viability
Weight	↑	↑
Body composition	↑ triceps mass ↓ fractional mass of several tissues	↑ triceps
Substrate utilization	↑ MG ↔ AIB	↑ MG ↔ AIB
Circulating metabolites	↑ amino acid ↓ cholesterol	↑ amino acid
<b>Placenta</b>		
Weight	↔	↔
Structure	↔	↑ development of exchange region
Hormone synthesis	↑ estradiol	↑ progesterone
Substrate uptake	↑ MG ↔ AIB	↑ MG ↔ AIB
<b>Mother</b>		
Weight gain	↔	↔
Body composition	↓ adiposity	↔
Circulating metabolites	↑ glucose	↑ glucose
Substrate utilization	Delay in plasma MG clearance ↑ MG and AIB	↔ MG ↑ AIB

\*\*previously determined by Prue Standen (Standen *et al.*, in preparation)

substrate availability for the fetus. IGF-II on the other hand, operates primarily through the placenta, to enhance placental development and improves its function and substrate supply to the fetus. Nevertheless, there is some degree of overlap between IGF actions, given that both IGFs appear to enhance maternal adaptation and improved placental transport function.

The discrete effects of IGF-I and IGF-II are speculated to stem from distinct interactions of the IGFs with various receptors. All three receptors, InsR (Kelly, Posner *et al.* 1974; Posner 1974), IGF1R and IGF2R (Chapter 4) are abundantly expressed by the guinea pig placenta at the time of treatment and are ubiquitously expressed by maternal tissues, permitting endocrine IGF actions. It is important to remember that the abundance of these receptors may also have been altered by maternal IGF treatment and that the guinea pig placenta may express InsR/IGF1R hybrid, as the human (Baillyes, Nave *et al.* 1997; Soos and Siddle 1989), which may have modulated IGF actions.

IGF-I has negligible affinity for IGF2R and InsR (De Meyts, Wallach *et al.* 1994; Jones and Clemmons 1995). Thus all effects of IGF-I treatment are likely to be mediated by IGF1R, particularly since this treatment also reduced maternal plasma IGF-II concentrations in mid pregnancy. IGF-II, on the other hand, can bind all three receptors, making it difficult to decipher which one is responsible for exerting the effects of exogenous maternal IGF-II. We aimed to define the contribution of IGF2R to the sustained impacts of IGF-II during pregnancy by administering Leu<sup>27</sup>-IGF-II, an analogue of IGF-II, which selectively interacts with IGF2R due to reduced binding affinity for IGF1R and InsR (Sakano, Enjoh *et al.* 1991) to the mother. Placental structural analysis revealed that many of the effects seen with maternal IGF-II treatment on placental labyrinthine development, were also observed in placenta from mothers administered Leu<sup>27</sup>-IGF-II. Like IGF-II, Leu<sup>27</sup>-IGF-II increased labyrinth proportion and volume, while reducing the weight of the interlobium. In terms of labyrinthine development, Leu<sup>27</sup>-IGF-II treatment was demonstrated to be more potent than IGF-II, as seen by increased volume densities of trophoblast and maternal blood spaces, increased total surface area of trophoblast for exchange and reduction in the thickness of the barrier for diffusion. This is consistent with the previously reported stimulation of IGF-II through IGF2R, on trophoblast migration and invasion (McKinnon, Chakarborty *et al.* 2000) and uterine angiogenesis and vascular remodelling (Herr, Liang *et al.* 2003). Leu<sup>27</sup>-IGF-II may have also promoted labyrinthine development indirectly by inhibiting activation of TGF- $\beta$ 1 activation at IGF2R (Godar, Horejsi *et al.* 1999; Leksa, Godar *et al.* 2005) in the placenta.



The observed placental structural changes following IGF-II and Leu<sup>27</sup>-IGF-II treatments, were predicted to enhance the capacity of the placenta to deliver substrate and are consistent with observed increases in methyl glucose transfer, fetal plasma total amino acid concentration, substrate utilization, growth and survival in late pregnancy. We are also aware that Leu<sup>27</sup>-IGF-II may have altered circulating IGF concentrations, or forced endogenous IGF-II to interact with IGF1R or InsR, both of which, have established roles in transport. Determining the circulating concentration of IGFs, as well as abundance of their cognate receptors in response to Leu<sup>27</sup>-IGF-II treatment, would help to better understand our observations.

There was largely no effect of maternal Leu<sup>27</sup>-IGF-II treatment on body composition and substrate utilization in the mother in late pregnancy. This suggests that the observed effect of IGF-II treatment of maternal System A amino acid uptake by maternal visceral and combined tissues, was mediated through IGF1R or InsR.

Further studies are required to define the downstream molecular pathways involved in maternal IGF control of fetal growth and survival. It is likely that the IGFs modulate the expression of molecules in the placenta involved in metabolism, transport, trophoblast differentiation, angiogenesis, blood flow regulation and maternal adaptation to pregnancy. Certainly, preliminary work in the laboratory, suggests that components of the placental renin-angiotensin system are altered in mid gestational guinea pigs, in response to maternal IGF supplementation (Standen *et al.*, in preparation). In addition, the IGFs may coordinate placental functional development with the environment of the mother and control fetal growth via the “nutrient sensor”, mTOR protein, signalling pathway which has established roles in the control of cell function and is expressed by the human placenta (Kim and Sabatini 2004; Kim, Sarbassov *et al.* 2002). Identification of differentially expressed genes and proteins within the placenta and maternal tissues, in response to earlier maternal IGF treatment, will further characterise and dissect the mechanisms and signalling pathways that transform placental function and hence promote fetal growth and survival late in gestation.

### ***7.1.3 Implications for human health***

The research in this thesis has increased our knowledge and highlighted the importance of maternal IGFs in their regulation of fetal growth and survival. Importantly, these studies suggest that elevating IGF-II abundance in the mother during early pregnancy may be used as a therapy for women at risk of pregnancy complications associated with placental dysfunction. Placental insufficiency has been

implicated in common and major disorders of pregnancy, including unexplained miscarriage, preeclampsia, intrauterine growth restriction and preterm birth. Combined, the latter three disorders combined, affect 1 in 5 pregnancies in Australia, can lead to fetal and maternal mortality and morbidity and are currently unpreventable. Furthermore, perturbed fetal growth increases the risk of poor health in adult life.

It would be interesting to determine whether early treatment of the mother with IGF-II can improve pregnancy outcome in animal models of placental insufficiency. A previous study has demonstrated that IGF-I, but not IGF-II, supplementation during early to mid pregnancy in guinea pigs, partially restored the effect of maternal under-nutrition on fetal growth, in mid gestation (Sohlstrom, Fernberg *et al.* 2001). Given that the anabolic impacts of IGF-II administration on fetal and placental growth became apparent in later gestation in the work of the current thesis, further investigative studies determining the sustained impacts of maternal IGF supplementation in this experimental model are required. Certainly future investigations that aim to identify protein targets which transduce maternal IGF-II actions in placental development and function, have enormous therapeutic potential and may pave the way for prevention of pregnancy complications associated with placental insufficiency.

The challenge now is to identify women at risk of developing placental dysfunction. Given the fundamental roles of IGF-II in orchestrating placental and fetal growth identified in the present thesis and work of others, we suspect that alterations in maternally derived or locally produced IGF-II actions in the placenta would predispose a mother to disease during pregnancy. This may arise from impaired IGF-II synthesis, abundance of IGFBPs and their regulatory proteases and expression of receptors and intracellular signalling molecules, as discussed in Chapter 1. These may originate from genetic alterations, caused by polymorphisms, defects in methylation of *Igf-2/Igf2r* and splice or allelic variations, or lifestyle factors, including diet and nutritional status, stress and oxygen availability at the fetomaternal interface, all of which can interact with each other.

It is reasonable to assume that if a deficiency of maternal circulating or placental synthesis of IGF-II in early gestation were a cause of poor pregnancy outcome, it would be associated with disorders of pregnancy that display placental perturbations. Finding support for this hypothesis in the literature is difficult, as studies are inconsistent and often investigate in late pregnancy, as discussed in Chapter 1. Thus, current efforts in the laboratory are focussed on identifying genetic and protein markers in parents and their babies in early pregnancy that predict how the placenta will develop and correlate with pregnancy outcome.

## 7.2 Conclusion

Analysis of the role of oxygen and IGF-II in first trimester human explants has revealed a novel interaction between these factors in the regulation of placentation. This work has suggested that, prior to the onset of maternal blood flow to the placenta, IGF-II mediates the low oxygen response to promote trophoblast outgrowth and invasion. In addition, work in guinea pigs has shown for the first time that, increasing IGF abundance in the mother in early pregnancy has a persistent effect on placental transport and nutrient allocation between the mother and conceptus, resulting in enhanced fetal growth and survival near term. While IGF-I treatment altered maternal body composition in late gestation, increased exposure of the placenta to circulating maternal IGF-II laid the foundation for subsequent optimal placental growth and function. This work has suggested that an increased abundance of maternal circulating IGF-II during early to mid pregnancy may provide a therapeutic avenue for preventing placental insufficiency, which is associated with common and major diseases of pregnancy.

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