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Placental Peroxisome Proliferator-Activated Receptor Gamma and Leptin Receptor in Human Pregnancy

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Placental Peroxisome Proliferator-Activated Receptor gamma and Leptin Receptor in Human Pregnancy

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Award of

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USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

ABSTRACT

Leptin is a 16kDa peptide hormone that has recently been implicated in human reproductive processes. Like other hormones, leptin exerts its influence through its receptor. Of the three major isoforms of the receptor (OB-Ra, OB-Rb, and OB-Re), OB-Ra (the dominant short form) has been implicated in transport processes within rat and human placentas, suggesting a role for it in implantation and pregnancy maintenance.

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that are a subfamily of nuclear hormone receptors. PPARs form heterodimers with another nuclear hormone receptor, RXRα, before binding to PPAR response elements (PPREs). Recently PPARγ/RXRα heterodimers have been implicated in trophoblast differentiation, thus playing a key role in human implantation and placentation.

The present study used Western Blot analysis to quantify the presence of Ob-R and PPARγ protein in human placental tissue from first and second trimesters, and at term (n=9, n=10, n=12 respectively). PPARγ and OB-Ra were found at all stages of pregnancy. Levels of OB-Ra were significantly lower in second trimester than in first trimester or at term ($p<0.01$, one-way ANOVA). Interestingly, two different molecular weight (MW) protein bands of PPARγ were found, one at ~ 55kDa and another at ~80kDa. The 80kDa PPARγ protein decreased from first to second trimester and then increased to maximal levels at term (F=14.437, $p<0.0001$, one-way ANOVA), and the 55kDa PPARγ protein was maximal in first trimester, fell in the second trimester, and remained at this level until term (F=3.793394, $p<0.05$, one-way ANOVA). Furthermore, the 80kDa protein was greater than the 55kDa at all stages of pregnancy (first trimester $p<0.005$, second trimester $p<0.0001$, term $p<0.0001$).

The presence of OB-Ra and PPARγ over the three stages of human pregnancy demonstrates they are important in implantation and pregnancy maintenance, and this may aid in our understanding pathological conditions such as miscarriage and intrauterine growth retardation (IUGR).

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i)   incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

(ii)   contain any material previously published or written by another person except where due reference is made in the text; or

(iii)   contain any defamatory material.

Signature____████████████_____

Date_____26 / 2 / 03_____

ACKNOWLEDGEMENTS

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ABBREVIATIONS

15D-PGJ$_2$	15-Deoxy-Δ 12,14-prostaglandin J$_2$
α	alpha
ACTH	adrenocorticotropic hormone
ANOVA	one-way analysis of variance
β	beta
BSA	bovine serum albumin
CBBG	coomassie brilliant blue G-250 dye
cm	centimetre
CTB	cytotrophoblastic cell
°C	degrees celsius
ddH$_2$O	double distilled water
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EEC	endometrial epithelial cell
EVCT	extravillous cytotrophoblast
FSH	follicle stimulating hormone
GH	growth hormone
g	grams
γ	gamma
hCACTH	human chorionic adrenocorticotropin
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
hCT	human chorionic thyrotropin
hGH-V	human growth hormone-V
HPG	hypothalamic-pituitary-gonadal
hPL	human placental lactogen
IgG	immunoglobulin G
IUGR	intrauterine growth retardation
kDa	kilodalton
L	litre

LH	luteinising hormone
LSD	least significant difference
M	molar = moles per litre
μl	microlitre
μg	microgram
mg/mL	milligram per millilitre
mL	millilitre
MMP	metalloproteinase
mRNA	messenger ribonucleic acid
MW	molecular weight
n	number
NaCl	sodium chloride
$NaH_2PO_4H_2O$	monobasic sodium phosphate
Na_2HPO_4	dibasic sodium phosphate
NaOH	sodium hydroxide
NK	natural killer
nm	nanometre
Ob	*obese* gene
OB-R	leptin receptor
p	significance
PBS	phosphate buffered saline
PDGF-AA	platelet-derived growth factor –AA
PlGF	placental growth factor
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR-responsive elements
PVDF	polyvinylidene diflouride
RT-PCR	real-time polymerase chain reaction
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
t	*t*-distribution
TGF	transforming growth factor
TIMP	tissue inhibitor of MMP
V	volt

VEGF	vascular endothelial growth factor
w/v	weight per volume
x g	times gravity

CHAPTER 1:

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Normal implantation and placentation are critical for a successful pregnancy. Approximately 70% of all pregnancies fail to reach full term (Norwitz, Schust, & Fisher, 2001). Of all pregnancies that are lost, 75% are due to failure of implantation. Not only is implantation important but also maintenance of the pregnancy after implantation is essential. The incidence of miscarriage after implantation is estimated to be 25 to 40% (Norwitz et al., 2001). "The maintenance of early pregnancy is inextricably linked with placental growth and differentiation" (Norwitz et al., 2001, p.1405).

Successful implantation is the end result of complex molecular interactions between the uterine endometrial epithelium and a blastocyst (Norwitz et al., 2001). The blastocyst actively participates in a complex dialogue with the endometrial lining during implantation and placental growth (Hill, 2001). Important molecules in this communication process include cytokines, growth factors, adhesion molecules, apoptotic factors and angiogenic factors (Hill, 2001; Simon, Dominguez, Remohi, & Pellicer, 2001). Of these, the hormone leptin and the group of nuclear hormone receptors known as peroxisome proliferator-activated receptors (PPARs) are potentially important factors for both implantation and pregnancy maintenance.

The hormone Leptin is a 16kDa peptide hormone and is a product of the *Obese* (*Ob*) gene. The main site of leptin production is adipose tissue and it was originally described as an adipocyte-derived signalling molecule, involved in the control of body weight and energy expenditure (Auwerx & Staels, 1998). Leptin exerts its effects through the leptin receptor (OB-R) (Tartaglia et al., 1995), and the mouse, rat and human placentas have been shown to express leptin and leptin receptor (Masuzaki et al., 1997; Senaris et al., 1997; Henson, Swan, & O'Neil, 1998; Smith & Waddell, 2002).

Recent studies have described important roles for leptin in reproduction and neuroendocrine signalling (Auwerx & Staels, 1998; Cunningham, Clifton, & Steiner, 1999; Moschos, Chan, & Mantzoros, 2002), and research has shown

that human placental tissue synthesizes leptin (Senaris et al., 1997), and leptin receptors are present in the human endometrium and decidua, potentially participating in the implantation process (Simon et al., 2001). Gonzalez et al. (2000) suggest that leptin and leptin receptors may be part of the embryonic-endometrial dialogue during human embryo implantation. Further evidence suggests that placental leptin may be crucial for angiogenesis, placental and fetal growth, and immunomodulation (Ashworth et al., 2000; Hoggard, Haggarty, Thomas, & Lea, 2001). Furthermore, leptin secretion has been demonstrated to be regulated by steroid hormones, and this may link leptin with mechanisms supporting pregnancy maintenance (Henson & Castracane, 2000).

PPARs comprise an important subfamily of the nuclear hormone receptor superfamily (Rosen & Spiegelman, 2001). PPAR gamma (γ) is a ligand-dependent nuclear hormone receptor that is involved in such pathways as adipogenesis, control of cellular differentiation and proliferation, and the maintenance of insulin sensitivity (Vamecq & Latruffe, 1999; Rosen & Spiegelman, 2001). Barak et al. (1999), found PPARγ to be crucial for successful placentation in mice. These researchers found that PPARγ deficiency severely compromises maternal-fetal exchange functions. Furthermore, PPARγ null embryos showed evidence of myocardial thinning, and PPARγ was demonstrated to be necessary for normal placental vascularisation (Barak et al., 1999).

1.2   SIGNIFICANCE OF THE STUDY

Infertility related to the process of implantation and poor placental growth is a major problem in obstetric medicine, and implantation failure and miscarriage are considered to be major biological causes of the low reproductive rate in humans (Bulletti, Flamigni, & Giacomucci, 1996). There are variations in both placental structure, and the process of embryo-maternal interactions between species, and animal models are therefore often unreliable, and their relevance to human implantation processes are unclear (Norwitz et al., 2001). Human placental tissue will be used in this study in order to try and

overcome some of these limitations, and identify the stage of human pregnancy in which PPARγ and OB-R are likely to have important roles. No known studies to date have compared PPARγ expression in normal human placental tissue over the three trimesters of pregnancy, and only a few studies have quantified the leptin receptor protein throughout human pregnancy (Henson et al., 1998; Bodner et al., 1999).

It is necessary to understand the complex molecular mechanisms behind normal human implantation processes so that factors which may be involved in pathological conditions such as implantation failure, miscarriage and intrauterine growth retardation (IUGR) may be identified. This may lead to methods of improving the likelihood of a successful birth.

1.3   EXPERIMENTAL OBJECTIVE

The overall objective of this thesis was to use Western Blot analysis to examine OB-R and PPARγ protein expression over the course of human pregnancy, to test the hypotheses that:

1.   PPARγ proteins are present in human placental tissues throughout pregnancy.

2.   OB-R and PPARγ protein levels change throughout pregnancy.

3.   PPARγ and OB-R proteins have a role during the process of implantation and pregnancy maintenance.

1.4   EXPERIMENTAL DESIGN

Human placental tissue was collected through first and second trimesters and at term of pregnancy. PPARγ protein and OB-R protein were measured by Western Blot analysis at each stage.

CHAPTER 2:

LITERATURE REVIEW

2.1 IMPLANTATION

"Implantation of the blastocyst to the uterine wall represents a highly critical point during early human gestation" (Sherer & Abulafia, 2001 p. 3). Implantation involves four basic steps. (1) Apposition, or placement of the blastocyst into the uterine cavity; (2) attachment of the blastocyst to the endometrial surface epithelium; (3) invasion of the trophoblast into endometrial tissues; and (4) growth of placental tissues, including vasculogenesis and angiogenesis (Hill, 2001). Recent research demonstrates that both maternal and embryonic factors have roles in the implantation process (Hill, 2001; Sherer & Abulafia, 2001; Simon et al., 2001). The endometrium and blastocyst secrete hormones, cytokines, and growth factors to prepare the uterus for implantation. Dysregulation of any factors in this process may be involved in either failed implantation or pregnancy failure (Hill, 2001; Simon et al., 2001). Progesterone and human chorionic gonadotrophin (hCG) are secreted in large amounts by the syncytiotrophoblast and have a role in the trophoblast invasion (Bischof, Meisser, & Campana, 2000). Trophoblast invasion is due to an active biochemical process. According to Bischof, Meisser & Campana (2000), the invasive behaviour of cytotrophoblastic cells (CTBs) is due to their ability to secrete matrix metalloproteinases (MMPs), which are controlled by a tissue inhibitor of MMPs (TIMP), that inhibits their invasiveness. MMPs are a family of approximately seventeen human zinc-dependent endopeptidases collectively capable of degrading all components of the extracellular matrix (ECM) (Bischof et al., 2000). CTBs behave like metastatic cells except that they are only transiently invasive (during the first trimester). This invasiveness is normally limited to the endometrium and the proximal third of the myometrium (Bischof et al., 2000). Hence, there is both temporal and spatial regulation of invasion. Types of regulators involved in trophoblast-endometrial reactions include hormones, cytokines, growth factors, ECM glycoproteins, adhesion molecules, apoptotic factors, and angiogenic factors (Bischof et al., 2000; Hill, 2001; Simon et al., 2001). Angiogenesis is the development of new capillaries from existing vessels, and is essential for normal implantation and placentation (Sherer & Abulafia, 2001). Abnormal

angiogenesis has been implicated in disrupted implantation and placentation, as well as early pregnancy loss. Certain angiogenic growth factors are now known to be essential for angiogenesis, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF), platelet-derived growth factor-AA (PDGF-AA), transforming growth factor α (TGFα), and TGFβ (for review see Sherer & Abulafia, 2001). Leptin, leptin receptor and PPARγ have also been implicated in this process (Sierra-Honigmann et al., 1998; Xin, Yang, Kowalski, & Gerritsen, 1999)

2.2   DECIDUA

The decidua is the modified endometrium after implantation and is also the functional layer of the pregnant endometrium (Moore & Persaud, 1993). The decidua is formed following the endometrial reaction which occurs in response to blastocyst implantation and subsequently leads to decidualisation (Castellucci & Kaufmann, 1995). Decidualisation is characterised by enlargement of the endometrial stromal cells, an increase in the number and complexity of cytoplasmic organelles, and most importantly a corresponding increase in secretory and synthetic activities of the endometrium (Castellucci & Kaufmann, 1995).

There are three regions of the decidua; (1) the decidua basalis, which lies deep to the conceptus and forms the maternal component of the placenta, (2) the decidua capsularis; the superficial portion overlying the conceptus, which degenerates and disappears by about week 22 of pregnancy, (3) the decidua parietalis, which lines the uterine wall (Moore & Persaud, 1993).

The decidua is involved in the endometrial-embryonic dialogue during implantation processes, and it has been postulated to be involved in modulation of immune reactions during pregnancy. Lymphocytes make up about 10% of all cells found in the decidua and most of these are natural killer (NK) cells (Benirschke & Kaufmann, 1995).

2.3   PLACENTATION

Following implantation, trophoblast cells proliferate rapidly and invade the surrounding endometrium. As the endometrium is invaded, maternal blood vessels are trapped to form lacunae, which fill with maternal blood (Cunningham, MacDonald, Gant, Leveno, & Gilstrap, 1993). As lacunae join, a complicated lacunar network (labyrinth) is formed, which is partitioned by trophoblastic columns and comprised of the intervillous space and primary villous stalks, respectively (Cunningham et al., 1993), and is the beginning of a primitive uteroplacental circulation. The lacunar network later becomes the intervillous space of the mature human placenta (Moore & Persaud, 1993).

As invasion of the endometrium proceeds, two layers of trophoblast form; the inner cytotrophoblast, and the outer syncytiotrophoblast (Moore & Persaud, 1993). PPARγ is thought to be important in the process of trophoblast differentiation (see later). The syncytiotrophoblast (syncytium) is a multinucleated mass without recognisable cell boundaries. It produces secretions which enable the blastocyst to penetrate the endometrium (Moore & Persaud, 1993), and lines the intervillous space, extending over surfaces of all chorionic villi as well as over the inner surfaces of the chorionic and basal plates. The syncytiotrophoblastic surface is almost entirely covered in microvilli, that provide a vast maternal-fetal exchange zone. There have been many studies of the structural and functional significance of this layer, including research into surface enzymes, receptors for transplacental hormones, growth factors, and immunological factors (for reviews see Bischof et al., 2000; Norwitz et al., 2001). The cytotrophoblast is mitotically active, and forms new cells that migrate into the syncytiotrophoblast. Here the cells lose their membranes and become part of the syncytium (Moore & Persaud, 1993). Thus, cytotrophoblastic cells are the stem cells of the syncytium, supporting its growth and regeneration (Castellucci & Kaufmann, 1995).

Oxygenated blood and nutrients enter the lacunae from maternal spiral arteries, and deoxygenated blood and waste products are removed via the

veins of the uterus (Moore & Persaud, 1993). A fully mature maternal blood circulation through the placenta may not develop until the end of the first trimester, at about 10 to 12 weeks of the pregnancy (Johnson & Everitt, 2000).The maternal and fetal circulations pass close to each other but do not actually mix. In the intervillous space maternal blood flows around the fine terminal villi containing fetal capillaries. Placental blood constitutes about 10% of the total maternal cardiac output by the end of the pregnancy (Johnson & Everitt, 2000).Slow blood velocity in the spiral endometrial arteries is thought to protect the early conceptus (pre-embryo) from being dislodged by spurts of blood, as well as giving plenty of time for the exchange of metabolites at the placental interface. A similar slowing of the blood occurs on the fetal side of the circulation, where there is a massive expansion in cross-sectional area of blood vasculature due to a profusion of vascular branching, and terminal capillary dilations (Johnson & Everitt, 2000). Nutrition of the conceptus is initially histiotrophic (via uterine secretions and decidua), and changes to haemotrophic as the placental blood supply develops (Johnson & Everitt, 2000).

2.4   PLACENTAL GROWTH

As pregnancy progresses, the placenta grows more rapidly than the uterus (Craven, Zhao, & Ward, 2000). The percentage of decidual surface area occupied by the placenta increases from 3% in the first trimester to 26% by the end of the pregnancy (Craven et al., 2000). The diameter of the placenta grows from approximately 5 cm at 11 weeks, to 28 cm at 38 weeks (Craven et al., 2000). Growth in thickness of the placenta continues until about the eighteenth week of pregnancy with the increase in thickness being due to branching of the stem villi (Moore & Persaud, 1993). Craven, Zhao, & Ward (2000) propose that floating villi implant in decidual veins during early pregnancy at sites distant from the existing placental bed, contributing to the process of lateral placental growth. Thus, areas of decidua marginalis become decidua basalis.

2.5 THE MATURE HUMAN PLACENTA

The placenta is discoid in shape, with an average diameter of 22 centimetres. It is approximately 2.5 centimetres thick at the centre and weighs on average 470 grams (Benirschke & Kaufmann, 1995).It is 'haemomonochorial', meaning that the trophoblastic surface is in direct contact with maternal blood (Johnson & Everitt, 2000). It is also described as a 'chorio-allantoic' placenta, which is one that develops from the blastocyst wall and becomes fully vascularised from the allantois (the primitive embryonic bladder) (Johnson & Everitt, 2000).

2.5.1 Placental Structure

The placenta is both a fetal and maternal organ and is made up of the chorionic plate (fetal portion), and the basal plate (maternal portion), which together enclose the intervillous space (Benirschke & Kaufmann, 1995). The maternal surface has a 'cobblestone' appearance due to cotyledons (lobes) visible on its surface and grooves on the surface are formed by placental septa. The maternal surface is covered with maternal decidua basalis (Johnson & Everitt, 2000).

The fetal surface is covered with amnion and attached to the chorionic plate. Chorionic villi, which are vascularised with fetal blood project from the chorionic plate into the intervillous space. Here they are bathed in maternal blood which circulates through the intervillous space (Benirschke & Kaufmann, 1995). Some villi extend from the chorionic plate to the decidua and serve as anchoring villi (Cunningham et al., 1993). Where the intervillous space disappears at the placental margins, the chorionic plate and the fetal plate fuse to form the chorion laeve (smooth chorion) (Benirschke & Kaufmann, 1995).

2.6 VILLI

Anchoring villi (stem chorionic villi) anchor the chorionic sac and the placenta to the decidua basalis, and the villous chorion is attached to the decidua basalis by the cytotrophoblastic shell (Moore & Persaud, 1993). Within villi,

fetal vessels are connected to the fetal circulatory system via the chorionic plate and the umbilical cord (Benirschke & Kaufmann, 1995).

All villi have the same basic structure throughout the placenta, although individual villi have been shown to have differing functional specifications. Most fetal-maternal exchanges occur in placental villi, as well as the many metabolic and endocrine activities of the placenta (Castellucci & Kaufmann, 1995). Within villi, fetal vessels are connected to the fetal circulatory system via the chorionic plate and the umbilical cord (Benirschke & Kaufmann, 1995).

2.6.1   Development of Villi

Villi can be first distinguished by about the twelfth day after fertilisation (Cunningham et al., 1993). These primary chorionic villi are finger-like projections of the chorion (Moore & Persaud, 1993). Secondary chorionic villi are formed early in the third week, when mesenchyme grows into the primary chorionic villi and forms a core of loose connective tissue. Villi at this stage cover the entire surface of the chorion (Moore & Persaud, 1993). Tertiary villi (stem villi) are formed when angiogenesis occurs in the mesenchymal cells (Cunningham et al., 1993). The blood vessels soon become connected with the embryonic heart, and by the third week embryonic blood is circulating through the capillaries of the chorionic villi (Moore & Persaud, 1993). Both fetal and maternal blood vessels are now functional and placental circulation is established (Cunningham et al., 1993).

Until the eighth week, chorionic villi cover the entire surface of the chorionic sac. As the sac grows, the blood supply is reduced to a large proportion of villi that eventually degenerate, producing the smooth chorion (chorion laeve) (Moore & Persaud, 1993). At the same time, villi associated with the decidua basilis (maternal component of the placenta) rapidly increase in number, branch profusely, and enlarge. This area becomes the villous chorion (chorion frondosum) and forms the fetal component of the placenta (Moore & Persaud, 1993).

2.6.2 Histological Structure of Villi

The layers of a villus externally to the core consist of syncytiotrophoblast, villous cytotrophoblast (Langhan's cells), trophoblastic basement membrane, and finally the stromal core of the villus (Castellucci & Kaufmann, 1995). As described previously the syncytiotrophoblast is a continuous, multinucleated layer that lines the intervillous space. It does not consist of individual cells, and it is viewed as a single continuous structure (Moore & Persaud, 1993).

Cytotrophoblastic cells (Langhan's cells) lie between the syncytiotrophoblast and the basement membrane. As mentioned earlier they are the stem cells of the syncytium, supporting its growth and regeneration. In early pregnancy, this layer is nearly complete but later becomes discontinuous, as survival of the syncytium depends on cytotrophoblastic fusion. Basement membrane surrounds the stromal core of the villi. Its main function is to support the trophoblastic epithelium. The villous stroma at the core of the villi is composed of various types and amounts of connective tissue fibres, ground substance, and fetal blood vessels (Castellucci & Kaufmann, 1995). Types of fixed connective tissue cells in the villous stroma include; mesenchymal cells, reticulum cells, fibroblasts, myofibroblasts, Hofbauer cells, mast cells and plasma cells (Castellucci & Kaufmann, 1995).

2.7 PLACENTAL FUNCTION

The placenta is an endocrine organ that performs many diverse functions. Firstly, it provides hormonal support for the developing embryo. Hormones synthesised by the placenta include human chorionic gonadotropin (hCG), human chorionic thyrotropin (hCT), human chorionic adrenocorticotropin (hCACTH), progesterone, estrogen, relaxin, somatomammotrophins including human placental lactogen (hPL), human growth hormone (hGH-V) and prolactin (Benirschke & Kaufmann, 1995; Martini & Welch, 1998). Secondly, it provides for the exchange of materials across the placental interface by various transport processes. These materials include gases, electrolytes, maternal hormones, waste products (urea, uric acid, bilirubin),

water, glucose, steroid hormones, as well as drugs and infectious agents. Thirdly, it performs many fetal metabolic functions as a substitute for the still immature fetal organs, including gas transfer, excretory functions, hematopoiesis, synthetic and secretory functions, and catabolic and resorptive functions (Benirschke & Kaufmann, 1995).

2.8 INCIDENCE OF MISCARRIAGE

The probability of conceiving a clinically recognised pregnancy within a given cycle has been estimated to be about 25%. However, this becomes closer to 32% when unrecognised pregnancies are included (Wilcox et al. cited in Cramer & Wise, 2000, p. 333). This suggests that a number of infertile women may actually be experiencing recurrent unrecognised pregnancy losses (Cramer & Wise, 2000). The incidence of infertility is estimated to be 10 to 15% of all couples (Reyniak cited in Bulletti et al., 1996, p.119), and some of this is due to implantation failure and early pregnancy loss.

Fertilization and the earliest development of the embryo do not lead inevitably to a sustained pregnancy and successful birth. Loss of the human conceptus is common and can occur at any stage of pregnancy (Johnson & Everitt, 2000). Only 50 to 60% of all conceptions advance beyond 20 weeks of gestation (Norwitz et al., 2001). Evidence suggests that up to 50% of all conceptions produce zygotes that do not survive the cleavage stage (during the first week after fertilisation), resulting in failure of implantation. Of those embryos that have faulty implantation the average survival time is 8 weeks, with 20% failing to complete 20 weeks of development (Martini & Welch, 1998). Failed embryo implantation is the cause of 75% of lost pregnancies, which as a result often remain undetected. It is also a major cause of those embryos lost in assisted reproduction (Norwitz et al., 2001).

Reproductive failure can also be due to spontaneous abortion (miscarriage) or recurrent abortion. Recurrent abortion is usually diagnosed as at least three consecutive miscarriages (Bulletti et al., 1996). The incidence of pregnancy loss after successful implantation is estimated to be 25 to 40%

(Norwitz et al., 2001). The cumulative result of losses both before and after implantation is that only 15 to 20% of human conceptions survive to a successful birth (Johnson & Everitt, 2000).

2.9   AETIOLOGY OF MISCARRIAGE

Chromosomal anomalies are responsible for miscarriage in at least 50% of all clinically recognised pregnancies, (Bulletti et al., 1996; Cramer & Wise, 2000; Johnson & Everitt, 2000). It has been suggested however, that the largest single cause of pregnancy loss is through failure of implantation, which has been estimated to be as high as 78% in humans (Webb & Glasser cited in Bulletti et al., 1996, p. 120). Conceptus loss before implantation has been attributed to a variety of reasons (for reviews see Bulletti et al., 1996; Cramer & Wise, 2000).

Although a large percentage of recognised pregnancies fail due to chromosomal anomalies, it is not known to what extent these anomalies influence conceptus loss in unrecognised pregnancies. These pregnancies possibly represent the highest percentage of human reproductive failure (Bulletti et al., 1996; Norwitz et al., 2001). An overwhelming 40 to 60% of miscarriages are of either uncertain or unknown origin (Bulletti et al., 1996; Johnson & Everitt, 2000).

2.10   LEPTIN AND LEPTIN RECEPTOR (OB-R)

Leptin is a 16kDa hormone and is a product of the *obese* (*Ob*) gene. It was first described as an adipocyte-derived signalling factor involved in the control of body weight and energy expenditure (Auwerx & Staels, 1998; Hoggard, Crabtree, Allstaff, Abramovich, & Haggarty, 2001). The main site of leptin production is adipose tissue (Zhang et al., 1994), but it is also present in other tissues. It has been found to be present in rat brain and pituitary (Morash, Li, Murphy, Wilkinson, & Ur, 1999; Jin et al., 2000), the human pituitary gland (Jin et al., 1999), the human and rat stomach (Bado et al., 1998; Mix et al., 2000), and human mammary epithelial cells (Smith-Kirwin et al., 1998). Masuzaki et al. (1997) and Senaris et al. (1997) were the first to

report that leptin is synthesised in and secreted from the human placenta. *In vitro* studies undertaken to measure leptin production by placental tissue found that it produces large amounts of this hormone (Linnemann et al., 2000). Recent studies have examined potential roles for placental leptin, including fetal growth, immunomodulation, haematopoiesis and angiogenesis (Auwerx & Staels, 1998; Sierra-Honigmann et al., 1998; Cao, Brackenhielm, Wahlestedt, Thyberg, & Cao, 2001; Hoggard, Haggarty et al., 2001; Moschos et al., 2002), and there is a large body of evidence indicating that leptin has roles in reproduction (Sabogal & Munoz, 2001; Moschos et al., 2002).

Observational studies have demonstrated that states of leptin excess, deficiency, or resistance, may be associated with abnormal reproductive function (For review see Moschos et al., 2002). Cheung et al. (1997) demonstrated that in female rats leptin acted to restore the normal timing of puberty following modest growth retardation imposed by food restriction. Food restriction had already been shown to delay the onset of puberty (Cheung et al., 1997). Similarly, when leptin was injected into normal prepubertal female mice it caused an earlier onset of reproductive function than in controls, suggesting that leptin acts as a signal triggering puberty (Chebab, Mounzih, Lu, & Lim, 1997). Animal experiments and observational studies indicate that a rise in leptin levels may be the earliest signal of the onset of puberty and may contribute to the activation of the hypothalamic-pituitary-gonadal (HPG) axis, resulting in an increase in sex steroid production (Moschos et al., 2002). Recent studies show that leptin influences the regulation of FSH, LH, ACTH, cortisol, and GH concentrations (Barash et al., 1996; Jin et al., 1999; Ozata, Ozdemir, & Licinio, 1999), and leptin gene expression itself is regulated by a variety of hormones, growth factors, and cytokines (for review see Moschos et al., 2002).

Leptin exerts its effects by interacting with its receptor (OB-R) located in the central nervous system and in peripheral tissues (Auwerx & Staels, 1998). The leptin receptor belongs to the class 1 cytokine receptor family, and has been found at all points along the HPG axis (Moschos et al., 2002), suggesting that leptin plays an important role in the modulation of the

reproductive axis (Cunningham et al., 1999). It has at least six splice variants (OB-Ra to OB-Rf), and the various isoforms have identical extracellular domains, with either long (~300 amino acids), or short (~30 amino acids) intracellular domains (da Silva, Bjorbaek, Uotani, & Flier, 1998; Cunningham et al., 1999). OB-Ra, OB-Rb and OB-Re have been identified in the rat and mouse placentas (Hoggard et al., 1997; Seeber, Smith, & Waddell, 2002; Smith & Waddell, 2002). OB-Rb is the dominant long form of the receptor and OB-Ra the dominant short form (Hoggard et al., 1997; Huang, Wang, & Li, 2001). OB-Re is the soluble form of the receptor and circulates in the plasma (da Silva et al., 1998; Huang et al., 2001). OB-Rb is considered to be the signalling form of the receptor (Ashworth et al., 2000), while OB-Ra is thought to participate in transporting leptin across the blood-brain barrier, and is expressed predominantly in the choroid plexus (Tartaglia, 1997). Smith & Waddell (2002) suggest that placental expression of OB-Ra, OB-Rb, and OB-Re is likely to mediate leptin transport in the fetus and placenta. They found that OB-Ra and OB-Re mRNA expression increased substantially in the period of maximal growth in the rat placenta (Day 16 to Day 22), whereas OB-Rb remained relatively stable (Smith & Waddell, 2002). The increase in OB-Ra was specific to the site of maternal-fetal exchange (the placental labyrinth zone) which they suggest is consistent with increased transport of maternal leptin to the fetus in late pregnancy (Seeber et al., 2002).

Several studies have localised OB-R to the syncytiotrophoblast of the human placenta by *in situ* hybridisation and immunohistochemistry. (Henson et al., 1998; Bodner et al., 1999; Ashworth et al., 2000; Hoggard, Haggarty et al., 2001). Bodner et al. (1999) comment that this indicates a role for the receptor in the growth of the placenta and/or fetus, and Henson et al. (1998) suggest it is indicative of the placenta's function as an endocrine organ. They hypothesise that there is likely to be a regulatory association between leptin and the steroid hormones, that may be basic to the maintenance of pregnancy (Henson & Castracane, 2000).

Expression of leptin and OB-Rs in the human ovary, oocyte, endometrium, embryo, and placenta, implicate them in implantation and early embryonic

development (Karlsson et al., 1997; Craven et al., 2000; Gonzalez et al., 2000; Kitawaki et al., 2000; Sabogal & Munoz, 2001; Simon et al., 2001). Kitawaki et al. (2000) were the first to demonstrate OB-R expression in the human endometrium. They found leptin receptor expressed in decidual tissue from early human gestation, and observed that whereas OB-R was expressed in the human endometrium, leptin was not. They suggest that leptin may play a specific role in interacting with other growth factors and cytokines during the implantation process (Kitawaki et al., 2000). Gonzalez et al. (2000), demonstrated leptin and its receptor to be expressed in secretory endometrium, and in cultured endometrial epithelial cells (EECs), and suggest that leptin is part of the embryonic-endometrial dialogue during the adhesion phase of human embryonic implantation. They hypothesise that the blastocyst and endometrium may communicate during implantation through leptin and its receptor. Simon et al. (2001), reported that human blastocysts and EECs secrete leptin, providing evidence to show leptin's presence and regulation at the human embryonic and endometrial surface.

Interestingly, in a study comparing peripheral maternal leptin levels with placental leptin levels in baboon pregnancy, Henson et al. (1999) found maternal leptin concentrations were positively correlated with gestational age, whereas placental leptin mRNA levels were negatively correlated with gestational age. These differences may imply changing roles for leptin with advancing pregnancy. The researchers comment that these changes are likely to be common to baboon and human pregnancies (Henson et al., 1999). Moreover, significantly lower concentrations of serum leptin observed in women who subsequently miscarried suggest that leptin may have a role in preventing miscarriage (Laird, Quinton, Anstie, Li, & Blakemore, 2001).

Malik et al. (2001) showed leptin to be a requirement for fertility, conception and implantation in the mouse, but observed that once pregnancy was established it was not required for maintenance of pregnancy. In contrast Mounzih, Qui, Ewart-Toland, & Chebab (1998) found that leptin was not essential for implantation, gestation, or fetal growth in mice, but it may be implicated in the regulation of maternal nutrition and metabolic adaptation

during pregnancy and lactation. They demonstrated a complete failure of lactation in leptin-deficient (*ob/ob*) female mice.

Many studies to date have used mouse and rat models to investigate the roles of leptin and its receptors in pregnancy. Due to the complexity of interactions between leptin, OB-R, and other molecules, the utilisation of animal models for the investigation of leptin action in human pregnancy has produced conflicting results. As leptin action may differ significantly between species, more human studies are warranted (Henson et al., 1998).

2.11 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (γ)

Peroxisome proliferator-activated receptors (PPARs) were first discovered in 1990 (Vamecq & Latruffe, 1999). They are ligand-dependent transcription factors belonging to the family of nuclear hormone receptors (Vamecq & Latruffe, 1999; Rosen & Spiegelman, 2001). There are three major isoforms of PPARs; PPARα, PPARβ, and PPARγ (Vamecq & Latruffe, 1999), that together control many cellular and metabolic processes (Wahli, 2002). PPARs have become an attractive target for pharmaceutical intervention in such metabolic diseases as obesity, diabetes, and hyperlipidaemia, and recently, they have been shown to have a role in epidermal differentiation and repair (Wahli, 2002).

PPARγ expression is dominant in white adipose tissue, and in immune cells in rodents and humans (Fajas et al., 1997; Vamecq & Latruffe, 1999; Clark et al., 2000; Padilla, Leung, & Phipps, 2002). PPARγ forms heterodimers with retinoid X receptors (RXRs) which then bind to PPAR-responsive elements (PPRE) within the promoters of PPARγ genes (Tarrade, Schoonjans, Guibourdenche et al., 2001). Moreover, PPARγ is involved in further complex interactions with various ligands, coactivators, and corepressors (Vamecq & Latruffe, 1999; Qi, Zhu, & Reddy, 2000; Rosen & Spiegelman, 2001), that have yet to be fully understood. Interestingly, Western Blot studies of PPARγ

protein to date have reported finding PPARγ protein at varying molecular weights (see Table 1).

Table 1: Suggested PPARγ Molecular Weights

Authors	Year	Tissue examined	PPARγ Molecular Weight (MW)
Capparuccia et al.	2002	human placenta	60kDa
Fajas et al.	1997	human adipose	60kDa
Padilla, Leung, & Phipps	2002	human B cells	80kDa
Tarrade, Schoonjans, Guibourdenche et al.	2001	human placenta	57kDa
Tarrade, Schoonjans, Pavan et al.	2001	human placenta	50kDa
Waite et al.	2000	human placenta	55kDa

Two isoforms of PPARγ are found in mice and humans, PPARγ 1 and PPARγ 2. Fajas et al. (1997) showed that the isoform PPARγ1 is the predominant form in humans. PPARγ has been described as the 'master regulator' of adipocyte differentiation (Vamecq & Latruffe, 1999, p.145), and it promotes cell differentiation in many cell types including hepatocytes, fibroblasts, myocytes, keratinocytes, monocytes, and macrophages. PPARγ has been implicated in cancer, atherosclerosis, type 2 diabetes, the regulation of insulin sensitivity and inflammatory responses (Vamecq & Latruffe, 1999; Rosen & Spiegelman, 2001; Wahli, 2002).

Barak et al. (1999) showed PPARγ to be necessary for normal placental development in the mouse, and found PPARγ deficiency caused an inability of fetal blood vessels to form a vascular network. These authors demonstrated that PPARγ deficiency interferes with both trophoblast

differentiation and placental vascularisation, leading to severe myocardial thinning and death of the embryo (Barak et al., 1999). Fetal-maternal exchange functions were severely compromised due to impaired vascularisation. This study revealed a novel developmental dependence of the heart on a functional placenta. Similar placental defects to those mentioned occurring in PPARγ null mice were observed in RXRα-deficient mice (Wendling, Chambon, & Mark, 1999). Recent studies have implicated PPARγ in trophoblast differentiation in humans (Schaiff et al., 2000; Tarrade, Schoonjans, Pavan et al., 2001). Furthermore, several studies have investigated the role of PPARγ in inhibiting angiogenesis. It has been found to be expressed at high levels in tumour endothelium, and researchers have demonstrated that PPARγ ligands can inhibit primary tumour growth through antiangiogenic effects (Xin et al., 1999; Panigrahy et al., 2002)

Recently, Cui et al. (2002), reported that the loss of the PPARγ gene in oocytes and granulosa cells led to impaired fertility. These researchers commented that although progesterone levels were decreased, and implantation rates reduced, they were not able to identify the exact mechanisms involved.

PPARγ has been localised by immunohistochemistry to human syncytiotrophoblasts and cytotrophoblasts (Schaiff et al., 2000; Waite et al., 2000; Tarrade, Schoonjans, Pavan et al., 2001; Capparuccia et al., 2002; Wang, Fujii, & Knipp, 2002). Only a few studies have quantified PPARγ protein in placental tissue in human pregnancy. Wang et al. (2002) observed PPARγ in rat and human placentas. Tarrade, Schoonjans, Pavan et al. (2001) measured PPARγ in first trimester placentas only, and Schaiff et al. (2000) in term placentas only. Recently, Capparuccia et al. (2002) observed PPARγ protein expression in early pregnancy and at term. These researchers found no significant quantitative difference in PPARγ expression between first and third trimester placentas. Their results demonstrated that PPARγ expression was downregulated in gestational trophoblastic diseases such as choriocarcinoma and hydatidiform moles, reinforcing earlier findings

that PPARγ is involved in the differentiation and invasiveness of placental trophoblast cells (Barak et al., 1999; Schaiff et al., 2000; Tarrade, Schoonjans, Pavan et al., 2001). Further studies are needed to elucidate the role of PPARγ over the course of human pregnancy.

2.12   PPARγ - OB-R INTERACTION

Yamauchi et al. (2001) observed a potential interaction between leptin and PPARγ/RXRα heterodimers. The authors were examining the roles of these receptors in insulin sensitivity, and reported that increases in PPARγ/RXRα activity were associated with decreased serum leptin levels (Yamauchi et al., 2001). Indeed this may imply a similar pattern of expression in placental function, and could be linked to recent evidence that PPARγ/RXRα heterodimers and OB-Ra are both involved in transport processes within the placenta (Seeber et al., 2002; Smith & Waddell, 2002; Wang et al., 2002).

2.13   SUMMARY

Human embryo implantation and placental growth are vital to achieving a successful birth. A deeper understanding is needed of the complex interactions between molecules at the fetal-maternal interface. Recent research indicates that leptin receptors and PPARγ appear to have essential roles in these communications. Deciphering these may contribute to reducing the high incidence of unexplained infertility and miscarriage, by providing possibilities for early clinical interventions in either pre-pregnancy or pregnancy itself.

CHAPTER 3:

MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

Ethics approval was obtained for this study from Edith Cowan University Faculty of Communications, Health and Science Ethics Sub-Committee for the conduct of Human Research, and the King Edward Memorial and Princess Margaret Hospitals Institutional Ethics Committees (EC 02-229).

3.2 TISSUE COLLECTION

Thirty-one samples of placental tissue were collected in total. Placental tissue was obtained following caesarean deliveries, at term (n=12), from King Edward Memorial Hospital, Subiaco, Western Australia. First trimester placental tissue (n=9), was collected following elective terminations at Nanyara, Rivervale, Western Australia, and second trimester placental tissue (n=10), was collected following elective terminations from Marie Stopes International, Midland, Western Australia. Term placentas were examined and weighed prior to cutting several small samples ($\sim$0.5cm^3) from the maternal surface which were then put into 1.5mL Eppendorf tubes, snap frozen on liquid nitrogen, and stored at -80°C for later Western Blot analysis. Due to the sensitive nature of, and the small amounts involved, first and second trimester placental tissues were not examined or weighed. However, as with term placentas, tissue was collected in 1.5mL Eppendorfs, snap frozen on liquid nitrogen, and stored at -80°C for subsequent Western Blot analysis. A patient information sheet was given to all patients and signed consent was obtained prior to collection (see Appendices 1 and 2 for the Form of Consent and Patient Information Sheet). First trimester placentas were from gestational weeks 6, 7 (x4), 8 (x2), 9, and 11. Second trimester placentas were from gestational weeks 13 (x2), 14, 15 (x3), 16, 17, 19 and 20.

3.3 PROTEIN EXTRACTION FOR WESTERN BLOT ANALYSIS

Protein was extracted from placental tissues following a standard laboratory protocol. Placental tissues were homogenised in RIPA Lysis Buffer (See Solutions and Buffers for Western Blot in Appendix 4) using a Polytron PT

1200 homogeniser ((Kinematica, Switzerland). One tablet of Complete, EDTA-Free Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Mannheim, Germany), and 50µl of 0.1M sodium orthovanodate were added to 50µl of RIPA Lysis buffer immediately prior to homogenisation. The cell lysate was then transferred to 1.5mL Eppendorf tubes and centrifuged at 11 000 x g at 4°C for 25 minutes. Following centrifugation the supernatant was collected, aliquoted into 1.5mL Eppendorf tubes, and stored at -20°C. A preliminary study found this protein fraction to be suitable for both leptin receptor and PPARγ detection.

3.3.1   Bradford Protein Assay

The Bradford protein assay was used to determine the total protein content of the placental tissue protein extracts. This assay is a dye-binding assay in which a colour change of dye occurs in response to various concentrations of protein. It works by the action of Coomassie Brilliant blue G-250 dye (CBBG). The absorbance maximum for a solution of CBBG shifts from 465nm to 595nm when binding to protein occurs.

3.3.1.1   Reagents

Bio-Rad Protein Assay Dye Reagent Concentrate and Bio-Rad Protein Assay Standard II Lypophilised bovine serum albumin (BSA) were purchased from Bio-Rad Laboratories, Hercules, USA.

3.3.1.2   Preparation of Standard Curve

The dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts ddH_2O and then filtered. Reconstituted BSA was mixed with ddH_2O to a concentration of 1mg/mL. Cuvettes were set up in duplicate, and a blank containing ddH_2O was prepared (see Table 3 in Appendix 3).

3.3.1.3   Preparation of Protein Samples

Protein samples were taken out of -20°C storage, thawed on ice, diluted in ddH_2O, and prepared in duplicate. Preliminary studies were performed to determine the correct dilution factor. Dilutions of 40x, 100x, and 250x were

tested and a dilution factor of 100x was found to be suitable. However, readings from eight samples did not fall within the standard curve and were then diluted 200x which gave an acceptable concentration.

The 100x dilution was prepared by diluting 5μl of protein sample in 495μl ddH$_2$O and the 200x solution was prepared by adding 5μl of protein sample to 995μl ddH$_2$O.

3.3.1.4   Spectrophotometry

Semi-micro cuvettes, polystyrene, 1cm path, 1.5mL, and a SmartSpec 3000 spectrophotometer were purchased from Bio-Rad Laboratories, Hercules, USA. Cuvettes for standard curve and cuvettes containing diluted protein samples were vortexed and incubated at room temperature for at least five minutes before reading in the spectrophotometer. The spectrophotometer was set to 595nm. The assay is monitored at 595nm and thus measures the CBBG complex with the protein.

3.4   WESTERN BLOT ANALYSIS

3.4.1   Reagents

The Kaleidescope Prestained Standard (broad range, #161-0324), and Prestained SDS-PAGE Standard (high range, #161-0309);Criterion Precast gels 7.5% and 10% (for OB-R and PPARγ respectively); running buffer, and Laemmli sample buffer, were all purchased from Bio-Rad Laboratories, Hercules, CA, USA. The premixed transfer buffer and β-mercaptoethanol were from ICN Biomedicals, Aurora, Ohio, USA. Ponceau S solution was from Sigma Chemical Co, St Louis, MO, USA; and Tween 20 from Asia Pacific Specialty Chemicals Ltd, Seven Hills, NSW.

3.4.2   Equipment

The Criterion Electrophoresis Cell Tank, Criterion Blotter Tank, PowerPac 200 power supply, Criterion Gel Holder Cassettes, Criterion Gel/Blot Assembly Tray, Immuno-Blot polyvinylidene diflouride (PVDF) membrane,

filter paper and fibre pads (9.5x15.2cm), were all purchased from Bio-Rad Laboratories, Hercules, CA, USA.

3.4.3   Buffers and Solutions

Buffers and solutions for Western Blot analysis were made up following standard protocols, and are shown in Appendix 4.

3.4.4   Preparation of Samples for Gel Loading

Extracted protein samples were prepared for loading onto gels immediately prior to loading. A total volume of 20μl was loaded into each well.

3.4.4.1   Reagents and Solutions

β-mercaptoethanol was purchased from ICN Biomedicals Inc. Aurora, Ohio, U.S.A. RIPA Lysis Buffer and Laemmli sample buffer were made to standard protocols (See Appendix 4)

3.4.4.2   Calculation of Protein Extract to Load 100μg of Protein

For each protein sample, the protein concentration for that sample (spectrophotometer reading) was used to calculate the amount of protein (in μl) to load for 100μg. 5μl Laemmli sample buffer, containing 5% β-mercaptoethanol was then added to that volume of protein, to which was then added RIPA lysis buffer to a final volume of 20μl. β-mercaptoethanol is used to reduce disulphide linkages in solubilising proteins for gel electrophoresis (see Table 2).

Table 2: Preparation of protein sample for loading

Protein concentration (mg/mL)	Protein volume to load for 100μg (μl)	Laemmli Sample Buffer (μl)	RIPA Lysis Buffer (μl)	Final Volume (μl)
e.g. 40	2.5	5	12.5	20

Samples were then given a short vortex mix (~4 seconds) followed by a 6 second mini-spin. The protein samples, together with the appropriate

molecular weight marker (MW) were incubated at 55°C for 10 minutes prior to loading onto gels.

3.4.5 Loading the Gels and Electrophoresis

Protein (100μg) was loaded into each lane, and the first lane in each gel contained 12 μl of MW standard (broad range for PPARγ and high range for OB-R). The precast gels (10% for PPARγ and 7.5% for OB-R) were set up in the Criterion tank according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA.), and each gel was run at 80V for 20 minutes, and then at 150V for ~80 minutes, until the dye front reached the bottom of the gel.

3.4.6 Protein Transfer

Following electrophoresis, the gel was placed in transfer buffer for 15 minutes to equilibrate. The Immuno-Blot PDVF membrane was placed in 100% methanol for a few seconds, until translucent, prior to transferring it to transfer buffer to equilibrate. The Criterion Blotter transfer apparatus and gel/membrane sandwich were prepared according to the manufacturers instructions (Bio-Rad Laboratories, Hercules, CA, USA.), and the transfer was run for 60 minutes at 100V on ice.

3.4.7 Ponceau S Staining

Membranes were removed from the transfer tank and placed in a shallow dish filled with PBS and Tween. Ponceau S stain (4-6mL) was poured onto the membranes to visualise and check for the even loading and transfer of proteins. All dye was then removed from the membranes with multiple washes in PBS and Tween.

3.4.8 PPARγ and OB-R Immunoblotting

Membranes were blocked at room temperature in 0.01M PBS and Tween containing 5% non-fat milk powder for one hour, and incubated overnight at 4°C with primary antibody (rabbit polyclonal antibody PPARγ (H-100) SC-7196, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:200 in

blocking solution; or rabbit (OB-R PA1-053, Affinity Bioreagents, Inc, Golden, CO, USA), diluted 1:2000 in blocking solution. Membranes were then washed 5 times in 0.01M PBS and Tween, and incubated at room temperature for 1 hour in secondary antibody (goat anti-rabbit IgG conjugated, SC-2030, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:20 000 in blocking solution. Membranes were then washed for 6 x 5 minutes in 0.01M PBS and Tween, followed by one rinse of ~ 20 seconds in 0.01M PBS (without Tween).

3.4.9 Visualisation of Bands

Detection of bound antibody was performed with the enhanced chemiluminescence Western Blotting detection kit (Amersham Pharmacia Biotech UK Ltd). The SuperSignal West Pico Substrate solution (Pierce Chemical, Rockford IL, USA) was prepared by combining an equal volume of Luminol/Enhancer solution and stable peroxide solution (to 10mL total). Membranes were placed in the solution for 5 minutes with gentle shaking.

Membranes were then exposed to Hyperfilm ECL (Amersham Pharmacia Biotech UK Ltd) and Hypercassette 8" x 10" (Amersham Pharmacia Biotech UK Ltd) for 1-10 minutes and developed on an Agfa Scopix LR 5200 developer. Membranes were stored at 4°C on 0.01M PBS prior to stripping and reprobing.

3.4.10 Stripping and Reprobing the Membranes

Membranes were stripped by incubating in stripping solution (See Appendix 4) for 30 minutes at 70°C. They were then washed for 2 x 10 minutes in 0.01M PBS and Tween. The stripped membrane was then incubated (as before) in SuperSignal West Pico Substrate working solution and exposed to film. The membrane was considered stripped when no bands were visible after 10 minutes exposure.

3.4.10.1 Non-Specific Antibody Binding

To check for non-specific antibody binding, membranes were incubated overnight at 4°C in blocking solution without the primary antibody, and then proceeding as previously described (See section 3.4.8).

3.4.11 Quantification and Statistical Analysis

Bands on the immunoblot images were quantified using Scion Image analysis (Scion Corporation, USA). All data are expressed as mean ±SEM. Changes in quantities of PPARγ and OB-R over the three stages of pregnancy were determined by one-way ANOVA and Least Significant Difference (LSD) tests. Differences in quantities of the different molecular weights of PPARγ protein (80kDa and 55kDa) in each trimester were assessed by unpaired two tailed t-tests. A correlation analysis was performed to assess possible relationships between (1) weeks of gestation and 80kDa PPARγ, (2) weeks of gestation and 55kDa PPARγ, (3) weeks of gestation and OB-Ra, (4) 80kDa PPARγ and OB-Ra, and (5) 55kDa PPARγ and OB-Ra.

CHAPTER 4:

RESULTS

4.1 WESTERN BLOT ANALYSIS OF PPARγ PROTEIN

Western blot analysis showed the presence of both a high molecular weight
(MW) protein band of approximately 80kDa, and a less intense lower MW
protein band of approximately 55kDa over the three stages of pregnancy
(see Figure 1). No signals were detected in membranes that were not
exposed to primary antibody. The 80kDa band was more intense than the
55kDa band over all stages of pregnancy (first trimester t=3.66, p<0.005;
second trimester t=7.80, p<0.0001; term t=21.74, p<0.0001, unpaired two
tailed t-test: see Figure 2). Quantification of the 80kDa PPARγ protein signal
showed that it decreased from first to second trimester and then increased to
maximal levels at term (F=14.437, p<0.0001, one-way ANOVA; p<0.05,
p<0.001, p=0.0799, LSD test). The 55kDa PPARγ protein signal was
maximal in first trimester, fell in second trimester and remained at this level at
term (F=3.793394, p<0.05, one-way ANOVA; p<0.05, p<0.05, p=0.733, LSD
test).
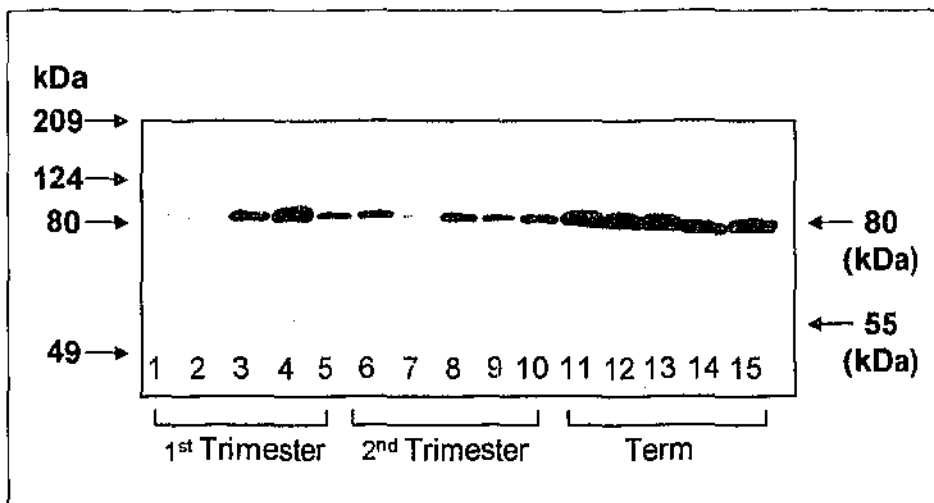
Figure 1. Western Blot of PPARγ protein in human placental tissue over the
course of human pregnancy. Arrows on the left indicate the MW marker.
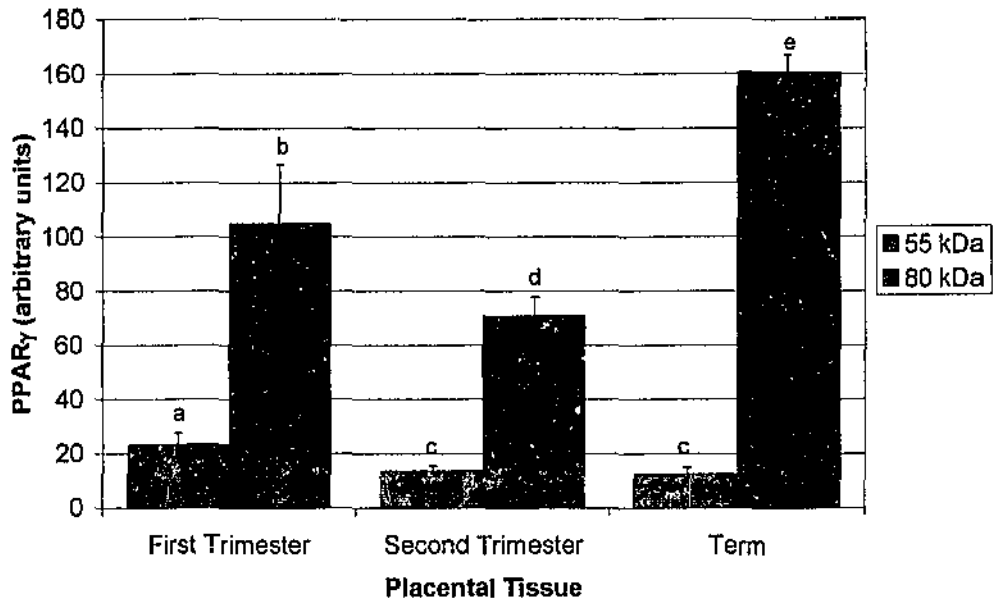
Figure 2. Quantification of Placental PPARγ protein (80kDa band and 55kDa band) over the three stages of human pregnancy. First trimester n=9, second trimester n=10, term n=12. Bars indicate mean ± SEM. At each stage of pregnancy those values without common notations differ significantly.

4.2 CORRELATION BETWEEN GESTATIONAL AGE AND PPARγ

Because of the wide range of gestational ages examined, a correlation analysis was performed to assess a possible association between the week of gestation and level of PPARγ. There was a significant positive correlation between the 80kDa MW bands and weeks of gestation (r=0.5894, r^2=0.3474, p=0.0005), and a significant negative correlation (r= -0.379, r^2=0.1436, p<0.05) between the amount of 55kDa protein and advancing gestation.

4.3 WESTERN BLOT ANALYSIS FOR OB-Ra

Western blot analysis showed the presence of a 100kDa band corresponding to the short form (OB-Ra) of the leptin receptor, at all stages of pregnancy (see Figure 3).
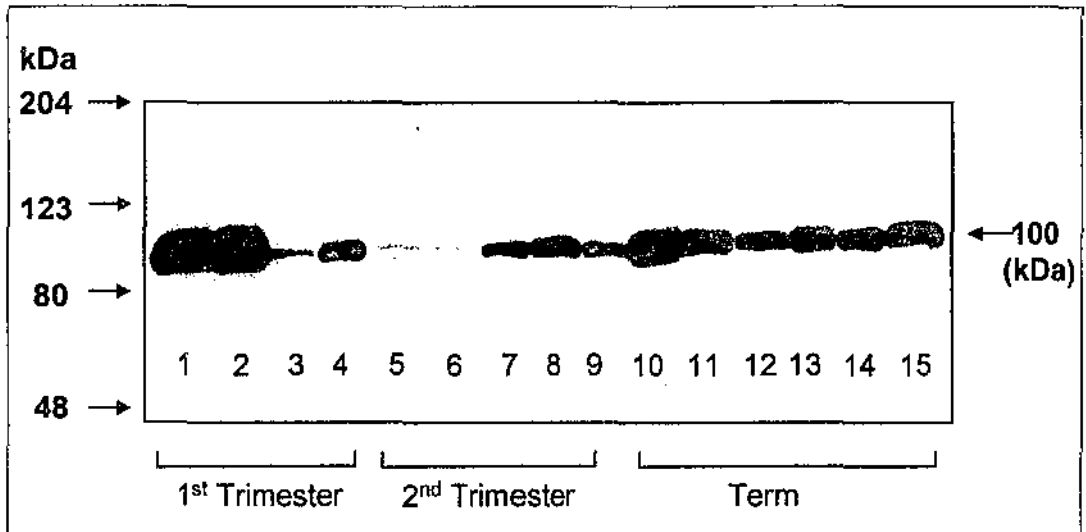
Figure 3. Western Blot analysis of OB-Ra protein in human placental tissues throughout human pregnancy. Arrows on the left represent the MW marker. 100kDa band is the OB-Ra protein.

Quantification of OB-Ra protein levels showed a significant decrease from first trimester to second trimester, and then a significant increase back to first trimester levels at term (F=5.73684, $p<0.01$, one-way ANOVA: see Figure 4).
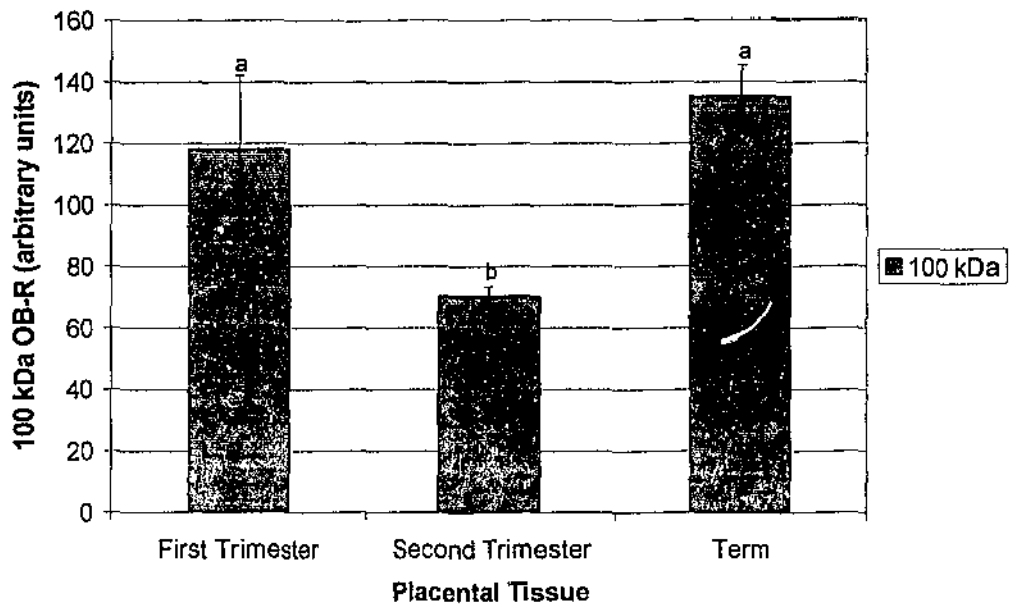
Figure 4. Quantification of Placental OB-R protein over the three stages of human pregnancy. First trimester n=9, second trimester n=10, term n=12. Data expressed as mean ± SEM. Bars without common notations differ significantly. OB-Ra protein levels changed significantly over the course of pregnancy (F=5.73684, $p<0.01$, one-way ANOVA). Levels were lower in second trimester compared to first trimester and term ($p<0.05$, $p<0.05$, $p=0.4070$, LSD test).

4.4 CORRELATION BETWEEN GESTATIONAL AGE AND OB-Ra

As with PPARγ, a correlation analysis was performed to assess for a possible relationship between gestational age and OB-Ra. There was no significant correlation between OB-Ra and gestational age (r=0.2914, r^2=0.0849, $p=0.1182$, two tailed t-test).

4.5 COMPARISON OF 80kDa PPARγ AND OB-Ra

Observations showed a similarity in the quantities of and pattern of the PPARγ 80kDa protein and OB-Ra over the three trimesters. To test if there was an association between the two proteins, a correlation analysis was performed showing a significant association (r=0.6937, r^2=0.4812, $p<0.0001$, two tailed t-test). The same test performed on the 55kDa PPARγ and OB-Ra did not show a significant correlation (r=0.232, r^2=0.0540, $p=0.2168$, two tailed t-test).

CHAPTER 5:

DISCUSSION

5.1 DISCUSSION

The experimental objective of this study was to examine OB-R and PPARγ protein expression throughout the course of human pregnancy. The present study has shown the presence of two different molecular weights of PPARγ protein, at 55kDa and 80kDa respectively, over the three trimesters of pregnancy, and OB-R protein at 100kDa representing the predominant short form of leptin receptor, OB-Ra.

There was a significantly different pattern of 80kDa and 55kDa MW proteins over the course of pregnancy, with the 80kDa protein increasing and the 55kDa protein decreasing as pregnancy advanced. The 80kDa PPARγ protein signal decreased from first to second trimester, then increased to maximal levels at term. The 55kDa PPARγ protein signal was maximal in first trimester, fell in second trimester, and remained at this level until term. Furthermore, there was a strong positive correlation between the 80kDa protein and weeks of gestation, and a strong negative correlation between the 55kDa protein and weeks of gestation.

Unlike previous studies (Fajas et al., 1997; Waite et al., 2000; Tarrade, Schoonjans, Guibourdenche et al., 2001; Tarrade, Schoonjans, Pavan et al., 2001; Capparuccia et al., 2002; Padilla et al., 2002), that have reported one band at varying molecular weights in human placental tissue, the present study showed evidence of two PPARγ protein bands, at 55kDa and 80kDa. These could possibly represent two PPARγ isoforms. Further studies are needed to fully characterise the two PPARγ proteins identified in this study. Molecular mechanisms involving PPARγ/RXRα heterodimers, and their interactions with ligands, coactivators, and corepressors are extremely complex (Vamecq & Latruffe, 1999; Qi et al., 2000; Rosen & Spiegelman, 2001), and are beyond the scope of this thesis.

The presence of PPARγ at all stages of pregnancy shown in this study suggests a functional role for PPARγ in implantation, and continuing throughout pregnancy. Recent studies on human placental tissue have

suggested a role for PPARγ in trophoblast differentiation and invasion (Schaiff et al., 2000; Tarrade, Schoonjans, Pavan et al., 2001; Capparuccia et al., 2002). A significantly higher level of 55kDa PPARγ protein was observed in the first trimester compared to second and at term, as well as a significantly higher level of 80kDa PPARγ protein in the first trimester compared to second. Early pregnancy is a crucial time for trophoblast differentiation and invasion. Furthermore, these processes are essential for successful implantation to occur.

OB-Ra protein was observed at all stages of pregnancy in the present study. The results show no significant differences between levels of OB-Ra in the first trimester and at term, similar to the observations of Henson et al. (1998) who also examined first trimester and term human placental tissue and found no difference. These authors however did not examine second trimester placentas, unlike the present study showed that OB-Ra levels decrease significantly in second trimester.

In the rat placenta, Smith & Waddell, (2002) found that OB-Ra protein expression increased significantly from Day 16 to Day 22 (the period of maximal growth; term = Day 23). However, it is difficult to ascertain whether Day 16 of rat pregnancy is equivalent to the first trimester or second trimester in human pregnancy. If Day16 of rat pregnancy is the equivalent to second trimester in human pregnancy then their findings support the findings of the present study.

The functional importance of leptin and its receptors in human implantation and pregnancy have been discussed recently in several studies, Ashworth et al. (2000), and Hoggard, Haggarty et al. (2001), both describe roles for leptin and its receptors in processes such as fetal growth, angiogenesis, haematopoiesis, immunomodulation, and in numerous complex molecular interactions at the fetal-maternal interface. The results of the present study suggest an important functional role for the short form of the leptin receptor in implantation and pregnancy. Higher levels of OB-Ra in first trimester and

at term than in second trimester, suggest a functional role for the receptor at these times. These roles are likely to be in angiogenesis in the first trimester, and in transport functions between the placenta and mother and/or fetus at term.

The present findings show a strong correlation between OB-Ra and PPARγ over all stages of pregnancy. Both PPARγ and OB-Ra have been implicated in angiogenesis (Sierra-Honigmann et al., 1998; Barak et al., 1999; Xin et al., 1999; Ashworth et al., 2000; Hoggard, Haggarty et al., 2001; Panigrahy et al., 2002) and placental transport functions (Vamecq & Latruffe, 1999; Seeber et al., 2002; Smith & Waddell, 2002; Wang et al., 2002). The present study raises the possibility of an interaction between OB-Ra and PPARγ during human embryo implantation and throughout pregnancy, in processes that are essential to implantation and placentation.

5.2   LIMITATIONS OF THE STUDY

The present study could have been enhanced by examining placental OB-R and PPARγ mRNA expression by RT-PCR analysis. This however was not feasible at this time due to difficulties encountered in obtaining placental tissues, and delays in laboratory equipment set up.

5.3   FUTURE DIRECTIONS

Future directions for this study are firstly to investigate the regulation of PPARγ and OB-R in human pregnancy, and secondly to examine possible interactions between them. The present study has contributed to our understanding of normal placental ex ession of OB-R and PPARγ, which may facilitate further studies of pathological conditions such as miscarriage and IUGR. An unexpected result in this study was the presence of two different molecular weight PPARγ protein bands throughout pregnancy (55kDa and 80kDa). Further characterisation of these bands is warranted.

5.4   CONCLUSION

In conclusion, the present study has demonstrated the presence of both OB-Ra and PPARγ protein over the three stages of human pregnancy. High levels of each protein, in early human pregnancy and at term, suggest they are important in human implantation and placentation as has been shown in animal models. Functional roles for them are likely to be in angiogenesis, placental transport processes and trophoblast differentiation. Furthermore, this study has highlighted a possible interaction between PPARγ and OB-Ra in pregnancy. Further studies will focus on elucidating their regulation, and investigating whether their downregulation is involved in pathological conditions such as miscarriage and IUGR.

CHAPTER 6:

REFERENCES

6.1 REFERENCES

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CHAPTER 7:

APPENDICES

7.1   APPENDIX 1

FORM OF CONSENT

I,

....

Given names   Surname

- have read the information explaining the study entitles **"Novel actions of leptin in implantation and placental function"**.

- have read and understood the information given to me. Any questions I have asked have been answered to my satisfaction.

- understand I may withdraw from the study at any stage and withdrawal will not interfere with routine care.

- agree that research data gathered from the results of this study may be published, provided that names are not used.

Dated....................... day of .... 20 ....

Signature ....

I, ... have explained the above to the
                    (Investigator's full name)

signatory who stated that he/she understood the same.

Signature ....

7.2 APPENDIX 2

<div align="center">

PATIENT INFORMATION SHEET

Novel actions of leptin in implantation and placental function

</div>

Poor fetal growth and implantation failure remain major problems in obstetric care. Successful implantation is where the embryo embeds itself in the lining of the uterus and establishes a placenta. It is estimated that among human conceptions that do not advance beyond midgestation, 75% are due to implantation failure, and low birth weight accounts for 10% of annual child health costs.

The establishment, growth and function of the placenta is of critical importance to the successful maintenance and completion of pregnancy. The placenta is effectively the lifeline of the growing fetus through its supply of nutrients, removal of wastes and coordination of hormone signals that help the fetus grow.

Among these signals a hormone called leptin, which is better known for its role in regulation of body weight has been identified as a crucial player in the control of fetal growth. Another molecule known as PPAR has recently been shown to be essential for implantation of the embryo into the uterus and the establishment of a normal healthy placenta. In this study we will examine leptin and PPAR in placental tissues from various stages of pregnancy and in cases of poor fetal growth and miscarriage. We will use standard laboratory procedures to measure the amount of these hormones in the placenta.

The tests will then be done using small samples (3-4) about the size of a thumbnail taken from the placenta after delivery. No information that can identify you will be kept for these samples. No genetic information is being studied. It is likely that the results will be published, but no way of identifying you will be possible. Placentas will be disposed of following standard KEMH procedures once the samples have been taken.

If you have any questions regarding this research project please contact:

Dr Peter Burton
Scientific Director
Concept Fertility Centre
Telephone: 93822388

If you have any complaints regarding the conduct of the study please contact the Executive Director of Medical Services at KEMH on 9340 2222

7.3 APPENDIX 3

Table 3: Standard curve for the Bradford Protein Assay

STANDARD No	PROTEIN CONCENTRATION (mg/ml)	PROTEIN STANDARD (µl)	ddH20	DILUTED PROTEIN DYE (µl)
1	0.00	0	25	975
2	0.10	2.5	22.5	975
3	0.20	5	20	975
4	0.40	10	15	975
5	0.60	15	10	975
6	0.80	20	5	975
7	1.00	25	0	975

(The linear range of the Bio-Rad Bradford Protein Assay for BSA is 0.2 – 0.9 mg/ml.)

7.4 APPENDIX 4

BUFFERS AND SOLUTIONS FOR PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

RIPA Lysis buffer

1.5M NaCl	100mL
1M Tris-HCl pH7.5	50mL
Triton-x-100	10mL
Deoxycholic Acid	5g
10% SDS	10mL

The volume was adjusted to 800mL with ddH_2O, the pH was brought to 8.0 with 4M NaOH, and then the volume further adjusted to 1L with ddH_2O.

Laemmli Sample Buffer

20% SDS	6.25mL
DTT	0.77g
2.5M Tris pH 6.8	1.2mL
0.5M EDTA	0.8mL
glycerol	1.75mL
1% Bromophenol blue	0.35mL

The volume was adjusted to 10mL and the pH was brought to 6.8. 5µl of β-mercaptoethanol was added to 95µl of sample buffer prior to loading the samples.

Running Buffer (1x Tris/Glycine/SDS)

0.025M Tris
0.192M Glycine
0.1% (w/v) SDS

100mL of 10x premixed buffer was added to 900mL of ddH_2O. The pH was brought to 8.3.

Transfer Buffer (Tris/Glycine)

0.025M Tris
0.0192M Glycine

1 packet of premixed buffer was added to 800mL methanol. The volume was adjusted to 4L with ddH_2O. The pH was brought to 8.3.

0.01M PBS

$NaH_2PO_4H_2O$	0.8g
Na_2HPO_4	5.5g
NaCl	35.7g

The volume was adjusted to 4L with ddH_2O. The pH was brought to 7.5. 4 mL of Tween was added to 4L of 01M PBS.

5% (w/v) Blocking Solution

Skim milk powder (5g) was added to 100mL 0.01M PBS and Tween.

Stripping Buffer

2.5M Tris-HCl pH 6.8	25µl
β-mercaptoethanol	9.98mL
SDS	20g

The volume was adjusted to 1000mL with ddH_2O.