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IMPLICATIONS OF INTERMITTENT FASTING ON PLACENTAL FUNCTION

Luke Sansby

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Masters of Philosophy (MPhil)

January 2015
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ABSTRACT

Introduction: Women who partake in Ramadan during pregnancy have lower placental weights. Intermittent fasting (IF) impacts upon asynchronous dietary cues and the entrained sleep cycle. The effects of IF on placental development and function have not been studied. Amino acid and calcium transport are key markers of a placenta’s nutrient transporting capabilities and are measured in pathological pregnancies to evaluate placental function.

Hypothesis: IF alters regulation of nutrient transporters in the placenta which may impact fetal wellbeing. These transporters may be under direct circadian control.

Methods: Rats were used to model either control or IF diet regimes during pregnancy. At term, fetal and placental weights were recorded and placental tissues homogenised. BeWo, a trophoblast-derived cell line, were cultured in different glucose concentrations to mimic asynchronous dietary cues. For both models protein content of key amino acid and calcium nutrient transporters (SNAT2, TRPV6, PMCA and Calbindin-D9K) and circadian machinery proteins (CLOCK and BMAL1) were quantified by western blotting. Analysis determined localisation and expression in response to dietary cues.

Results: Rat placental weight was reduced and fetal weight increased in IF versus control diets. Placental protein expression of TRPV6 and PMCA and SNAT2 was decreased whereas CLOCK protein was increased. In BeWo, both CLOCK and BMAL1 were localised to the nucleus and cytoplasm. Asynchronous dietary cues manipulate the expression of CLOCK and BMAL1 in BeWo. Serum shocking did not stimulate circadian oscillations in BeWo, but reduced CLOCK expression.

Discussion: The IF rat model mimicked effects on placental weight seen in humans during Ramadan. This coincided with aberrant nutrient transporter expression and circadian disruption. Placental reserve capacity may account for discrepancies in placental size and fetal weight seen in this model. Both models suggest that circadian machinery exists in placental trophoblasts. Transporter activity during circadian disruption should be investigated to reveal the effect of IF during pregnancy on placental function and fetal outcome.
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LIST OF ABBREVIATIONS (ALPHABETICAL ORDER)

11βHSD-2: 11βhydroxysteroid dehydrogenase type 2
ATP: Adenosine Triphosphate
BeWo: Immortalised trophoblast human cell line
BM: Basal Membrane
BSA: Bovine Serum Albumin
C: Control Diet Regime
Ca²⁺: Calcium Ion
CaBP: Calcium Binding Protein
CaSR: Calcium Sensing Receptor
CD9: Cell surface adhesion glycoprotein
CO₂: Carbon Dioxide
DH₂O: Distilled Water
DMEM: Dulbecco’s Modified Eagles Media
DMSO: Dimethyl Sulphoxide
DNA: Deoxyribonucleic Acid
E: Embryonic Day
EDTA: Ethylenediaminetetraacetic acid
ENU: N-ethyl-N-nitrosourea – a potent mutagen
EVT: Extravillous Trophoblasts
FBS: Fetal Bovine Serum
FGR: Fetal Growth Restriction
FMS: fms-like tyrosine kinase-1
hCG: Human Chorionic Gonadotropin
HLAG: Human Leukocyte Antigen G
hPL: Human Placent Lactogen
IF: Intermittent Fasting
IGF-II: Insulin-like Growth Factor-II
IUGR: Intrauterine Growth Restriction
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MVM: Microvillus Membrane
OD: Optical Density
PBS: Phosphate Buffered Saline
PBST: Phosphate Buffered Saline Tween
PCR: Polymerase Chain Reaction
PIC: Protease Inhibitor Cocktail
PMCA: Plasma Membrane Calcium ATPase
PNS: Post Nuclear Supernatant
PTH: Parathyroid Hormone
PTHrP: Parathyroid Hormone-Related Protein
PVDF: Polyvinylidenefluoride
RIPA: Radio Immuno Precipitation Assay
SCN: Suprachiasmatic Nucleus
SNAT1-4: Protein products of gene family Slc38a that form System-A Transporters
T: Time point
TBS: Tris Buffered Saline
TBST: Tris Buffered Saline Tween
TRPV: Transient Receptor Potential Vanilloid
TTO: Transcription Translation Oscillating
UK: United Kingdom
UV: Ultra Violet
WT: Wild Type
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CHAPTER 1: IMPLICATIONS OF INTERMITTENT FASTING ON PLACENTAL FUNCTION: A REVIEW OF THE LITERATURE
1.1 INTRODUCTION

Intermittent fasting (IF) is practiced widely across the globe for a number of reasons including religious festivals and supposed health benefits. As the impact of a period of IF has been further explored the focus has shifted towards effects it may have on pregnancy outcome. An adequate and regular source of nutrients is one key component to maintaining fetal growth and health; as such a period of fasting during gestation may have implications on fetal development. The placenta is the first organ to form during embryogenesis and plays a key role in fetal survival. One important element of placental physiology is its ability to efficiently transport nutrients from maternal to fetal circulation. Problems within the formation, development and function of the placenta have been frequently linked to poor pregnancy outcome with serious detrimental effects on fetal growth and wellbeing. Understanding the intricate relationship between placental development and function and fetal wellbeing is therefore a rapidly increasing area of research in both a clinical and basic science arena. In vivo and in vitro models are utilised to unravel placental physiology so nutrient transport is better understood. Both the disruption of the sleeping pattern and the change to dietary cues brought about by the intermittent nature of fasting periods may also have a role to play in the development of the placenta and fetal outcome via tissue specific circadian machinery located within the placenta.

The main aims of this literature review are three-fold; firstly to discuss intermittent fasting and its physiological effects on pregnancy outcome including the ability of asynchronous dietary cues and disrupted sleep patterns to disturb normal circadian oscillations. Secondly, to discuss the structure and function of key nutrient transporting zones in the placenta during healthy pregnancy. Finally this literature review will address the impact of circadian machinery and its ability to disrupt normal placental function
1.2 INTERMITTENT FASTING

1.2.1 THE CULTURE OF INTERMITTENT FASTING

Dietary restriction has been shown to have several health benefits; these include increased life span, delayed or prevented age related diseases, and improved functional and metabolic cardio-vascular health in rodents (Mager et al. 2006). Although most of these affects have been correlated with decreased caloric intake, another form of dietary restriction termed intermittent fasting (IF), in which not the calorie intake is reduced but the frequency by which calories are consumed, has shown similar results. Furthermore IF has been shown to impart beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake (Anson et al. 2003). The bodybuilding community advocates the IF principle after a range of studies reported benefits in fat metabolism, weight loss, cardio protection and insulin resistance (Smeets and Westerterp-Plantenga 2008; Klempel et al. 2010; Higashida et al. 2013).

IF is a key area of interest because of its relevance to real world situations; during the holy month of Ramadan it is obligatory for all adult Muslim’s to abstain from the consumption of food and drink from sun rise until sunset. Seasonal variation can alter the fasting hours of Ramadan because the Arabic calendar is dependent on the moon. Although pregnant women are exempt from the fasting period, many choose to participate on social grounds. Several studies have been conducted looking into the physiological effects this period of intermittent fasting has on the healthy non-pregnant body. It is widely reported that body weight and body mass index are reduced during the fasting period in both males and females (Ziaee et al. 2006; Lamri-Senhadji et al. 2009). Findings have also shown changes in blood pressure, lipids, apoproteins, fasting blood sugar, hormones and other biochemical parameters (Sarraf-Zadegan et al. 2000) indicating that fasting brings about sweeping changes in basic physiology.

The restriction of food is not the only challenging aspect of the fast. It has previously been reported that people practicing Ramadan are in a constant state of dehydration, suffer from
headaches and are sleep deprived (Leiper et al. 2003). It is also important to remember that Ramadan type fasting also brings about complications other than those associated with caloric restriction. The diurnal nature of humans is disrupted by the timing of the Ramadan fasts and thus changes typical feeding behaviour. Night time eating leads to a different sleep/wake cycle to that which is usually adhered to and also brings about asynchronous dietary cues, to which the body must adapt. It is well documented that disruptions to normal sleep wake cycles and eating patterns can cause a number of problems including desynchronisation of molecular clocks in both peripheral tissues and the central nervous system (Haus and Smolensky 2006).

1.2.2 IMPLICATIONS OF IF IN PREGNANCY

Although the physiological changes in the normal human body have been studied there is not a vast amount of information on the effects of intermittent fasting during pregnancy and the implications it may have on fetal health. Although pregnant women are exempt from the fasting period many still choose to participate fully on social grounds or participate in a limited capacity due to their pregnant state (Joosoph et al. 2004). Several studies have focused on physiological changes that occur during pregnancy and Ramadan. These studies have reported no differences between breast milk composition, a significant difference in fetal breathing movements during the fasting periods and finally, no difference between the uterine blood flow between control and fasting counterparts (Bener et al. 2001; Mirghani et al. 2004; Mirghani et al. 2007). More recently the placenta and pregnancy outcome during Ramadan have come under investigation.

A study looking into the effects of Ramadan fasting on pregnancy has found that babies who were exposed to maternal IF in utero during the second or third trimester of pregnancy had unaffected birth weight at term, however they had reduced placental weight at birth. This suggests that placentas used a reserve capacity to increase efficiency per gram of placental tissue and maintain adequate fetal growth despite their decreased size (Alwasel et al. 2010) (a phenomenon which has been observed in other environmental stressors of pregnancy
such as high altitude or maternal anaemia (Fox 1997)). The authors go on to tentatively suggest that the placental changes seen in Ramadan may be associated with fetal programming and health implications in the future. It should be noted however that monitoring human pregnancy during Ramadan is notoriously difficult. This study was limited by location, as the investigators only sampled one city, and by season, because the study was conducted over four years during which Ramadan only occurred in three different months. The mechanisms behind the changes witnessed during intermittent fasting remains unknown. However, it is important that we understand healthy placental physiology to be able to begin unravelling the placental reserve capacity and to what extent it can maintain fetal nutrition with regards to an altered maternal diet during gestation.

1.3 THE PLACENTA

1.3.1 DEVELOPMENT AND STRUCTURE

The human placenta is haemomonochorial with maternal and fetal blood circulations separated by a single syncytial membrane layer to facilitate transport of oxygen and nutrients from mother to fetus. Early placental development occurs in a five day differentiated blastocyst, in which trophectoderm derived placental cells, known as trophoblast cells, are the first fetal cells to form during implantation post blastocyst attachment to the endometrium. The trophoblasts cells form a uninucleate, single layer outer wall encasing the inner cell mass from which the embryo and umbilical cord derive (figure 1.3.1).

As time progresses trophoblastic cells continue to differentiate and form a double layer; the inner layer becomes the mononuclear cytotrophoblast layer whilst concurrently the outer maternal facing layer becomes the syncytiotrophoblast formed via fusion of cytotrophoblast cells to form a monocytoplasmic, multinuclear cell. The cytotrophoblasts act as “stem cells” that allow the process of fusion to continue throughout the gestational period for the renewal and maintenance of the syncytiotrophoblast layer.
During the early phase of placental maturation another sub class of trophoblastic cells known to proliferate from the cytotrophoblast cells, are the invasive extravillous trophoblasts (EVT). The EVT is responsible the invasion of the maternal endometrium and the manipulation of the spiral arteries to increase blood flow towards the fetus through the placenta (Forbes and Westwood 2010). Endovascular EVT migrate to the artery lumen and begin to degrade smooth muscle, these are soon replaced by trophoblast cells fixed in fibrinoid. Their function is to cause enlargement of the spiral arteries to increase maternal blood supply for the period of gestation (Kaufmann et al. 2003; van der Heijden et al. 2004), effects of which are not entirely reversed postnatally (figure 1.3.2).

The trophoblast lineage then plays a key role in the development of the villous tree structure. Small vacuoles start to form in the syncytiotrophoblastic mass. As the placenta matures, these mature to form complex branched “tree like” structures originating from chorionic (fetal) side and comprising of the syncytiotrophoblast on top of regenerating cytotrophoblast cells and stromal core containing fetal blood vessels (figure 1.3.3) (Benirschke 1973). The villous trees branch into the intervillous space from the fetal facing chorionic plate to come into direct contact with maternal blood flooding the intervillous space from the remodelled spiral arteries (figure 1.3.4). The proximity of maternal and fetal circulatory systems allows for the syncytiotrophoblast to become the key nutrient transporting zone in the human placenta (Burton 2006).
Figure 1.3.1: Five days after fertilisation the early embryo undergoes cell differentiation and structural changes to become the blastocyst. The inner cell mass is surrounded by a single cell layer of trophoblasts creating a blastocyst cavity.
Figure 1.3.2: The early development of trophoblast cells in implantation invasion into the endometrium. A) Implantation of blastocyst with outer trophoblast wall onto the endometrium. B) Extra villous trophoblasts (EVT) invade into the endometrium in search of maternal blood supply. C) Endovascular trophoblasts (arrows) break down smooth muscle lining of maternal spiral arteries causing widening of the artery lumen and increased blood flood to the placenta and intervillous space. Adapted from (Parham and Moffett 2013).
Figure 1.3.3: The syncytiotrophoblast functions as a protective barrier for the fetus whereas the extravillous trophoblasts invade maternal circulation. The villous stroma contains numerous cell types including placental macrophages, endothelial cells and fibroblasts. It is located directly beneath the cytotrophoblast layer. Adapted from (Forbes and Westwood 2010).
Five key stages of villous maturation occur throughout gestation to enable the placenta to comply with fetal demands and sustain fetal growth and development. Stem villi form from the chorionic plate and provide an anchoring structure for further branching. Following branching gives rise to mesenchymal, immature intermediate villi, mature intermediate villi and finally terminal villi. This progressive vasculature development is characterised by the sub-divisions of branching capillaries becoming finer at each step (Fox 1997). The development of the placenta sees an increase in efficiency and this is reflected in the villous branching structures. The terminal villi are recognised as the main functional transport sites of the placenta. The large surface area of the villous tree bathed in maternal blood, multinucleated syncytium and short distances between syncytium and fetal capillaries allow for efficient transport of nutrients and oxygen between mother and fetus whilst maintaining separate circulations. Aberrations of this stage of development have detrimental effects on fetal wellbeing with defects in placental angiogenesis and early placental development being linked to early onset intrauterine growth restriction (IUGR) (Kingdom 2000).
Figure 1.3.4: Placental villous tree structure. Fetal capillaries are located within the villous structures close to the interface between maternal and fetal circulation. The large surface area of the villous tree is bathed in maternal blood, multinucleated syncytium and short distances between syncytium and fetal capillaries allow for efficient transport of nutrients and oxygen between mother and fetus whilst maintaining separate circulations. Figure adapted from (Gallery 2011).
1.3.2 THE SYNCYTIOTROPHOBLAST

The syncytiotrophoblast is comprised of a multinucleated, monocytoplasmic cell, called the syncytium which is highly specialised in order to carry out its main functions, which are twofold. Firstly, the membrane is adapted to transfer nutrients, ions and waste products between maternal and fetal circulations. These adaptations include the presence of a microvillous brush border membrane facing the maternal circulation to increase surface area combined with the relocation of syncytial nuclei into aggregates to allow fetal capillaries to run close to the syncytial membrane to decrease diffusion distance and thus increase efficiency (Eaton 1993).

Secondly the synthesis of steroid and peptide hormones required for fetal development. Among these are human chorionic gonadotropin (hCG) and progesterone (Ogren 1994). Production of hCG begins early in pregnancy as its initial role is to stop the degradation of the corpus luteum. The corpus luteum then goes on to secrete the progesterone necessary to prevent early uterine contractions which would otherwise halt pregnancy via menstruation (Jameson and Hollenberg 1993).

Terminal villi, as described in section 1.2.1, are encompassed by the syncytiotrophoblast. The maintenance of syncytiotrophoblast is essential; thus there is a regenerative process that lasts throughout gestation which occurs via the proliferation of mononuclear cytotrophoblasts and the apoptosis of ageing syncytiotrophoblast nuclei.

1.4 PLACENTAL NUTRIENT TRANSPORT

Materno-fetal transport of nutrients, gases and waste products is not passively facilitated by the placenta but an actively controlled and mediated response to fetal demand signals. The placenta transports multiple nutrients, such as amino acids and calcium, and thus has adapted several systems.
**1.4.1 SYSTEM A AMINO ACID TRANSPORT**

Amino acid transport remains vital throughout the gestational period to provide both the components of proteins and the essential precursors for other non-protein substances. Amino acid transporter proteins are localised to both the microvillous membrane (MVM) and basal membrane (BM) of the syncytiotrophoblast. They differ in structure, function, dependency and specificity (Grillo et al. 2008). System A transporters include SNAT1, 2 and 4 which are specific for the transport of small zwitterionic, neutral amino acids (Johnson and Smith 1988). SNAT 3 and 5, however, are system N transporters the primary function of which is glutamine transport. This report will focus on the sodium dependent amino acid transport systems which are encoded by the Slc38a gene family which gives rise to three highly homologous protein isoforms of system A transporter, SNAT1, 2 and 4. Expression of the different isoforms is reported to be tissue specific, apart from SNAT2 which shows ubiquitous mammalian expression (Desforges et al. 2006).

It has been reported that SNAT isoforms 1, 2 and 4 are present in the placenta. More recently it has also been reported that SNAT3 is present in the placenta during early pregnancy (Novak et al. 2006; Yoshioka et al. 2009). SNAT4 mRNA (expression of the gene Slc38a4) is highest in the first trimester of gestation; in contrast it has been shown that protein expression is highest at term. This mirrors data suggesting all SNAT isoforms increase in expression between days 14 to 20, near the end of gestation, in the rat placenta (Novak et al. 2006). These data indicate there is an increased need for amino acids by the fetus and placenta in the final trimester. This this would be expected as it has previously been reported that fetal growth rates overtake those of the placenta in the final portion of pregnancy (Schneider 1996).

Maternal plasma levels of amino acids decrease by up to 25% below non-pregnant measurements as early as the first trimester of pregnancy which is converse to the 186% increase in seen in the intervillous space in the placenta (Camelo et al. 2004). Dietary changes to system A transport regulation are well documented, in particular changes to the
SNAT2 isoform. SNAT2 regulation is reported to be the most adaptable under nutritional challenge (Franchi-Gazzola et al. 1999). Adaptive regulation of system A can be shown in mesenchymal cells in the form of a marked increase in System A activity after a period of incubation in amino acid free media (Gazzola et al. 1972). Further to this, human fibroblasts which underwent a period of incubation in hypertonic conditions displayed an up regulation of system A activity which is attributed with restoring cell volume after shrinkage (Dall'Asta et al. 1994b). Indicating System A transport into the cell is important for retaining normal function after a period of stress. In addition to these studies it has been reported that in BeWo a period of amino acid starvation stimulates an increase in α methyl-amino isobutyric acid transport after as little as 1 hour post incubation, however this increase in activity was not mirrored in an increase in SNAT2 mRNA until 3 hours after incubation began and it took a further 3 hours before protein expression to significantly increased (Jones et al. 2006b).

1.4.1.1 System A and pregnancy complications

IUGR in human pregnancy is associated with a decrease in placental system A transporter expression; it has also been shown in animals that this down-regulation precedes IUGR (Jansson et al. 2006). Other evidence presented from animal studies reaffirms this conclusion. In one study, the system A transport axis was inhibited in the rat placenta by using a non metabolisable amino acid analogue and this in turn led to IUGR (Cramer et al. 2002). A second rat study reported altered function of system A amino acid transport during dietary change. A low protein maternal diet resulted in placental system A transport activity reduction by E19 of gestation. The same low protein model resulted in IUGR at term and the authors were able to conclude that the decrease in amino acid transport was a cause, rather than the result, of IUGR (Jansson et al. 2006).
1.4.2 Placental calcium transport and calcium homeostasis regulation

The ionic form of calcium (Ca$^{2+}$) is found ubiquitously in human tissues and serves many purposes; it mediates neurotransmission, enzyme and hormone secretion, apoptosis and cell cycle regulation (Clapham 1995). Fetal Ca$^{2+}$ demand during gestation puts considerable strain on the mother's calcium economy and during the final trimester of pregnancy the fetal Ca$^{2+}$ demand reaches its peak when two-thirds of the total Ca$^{2+}$ demand is transferred across the placenta. Although interactions between Ca$^{2+}$ homeostasis and Ca$^{2+}$ transport are complex it is known that disruptions to Ca$^{2+}$ homeostasis throughout the course of gestation can lead to an increased risk of developing osteoporosis later in life (Tobias and Cooper 2004). This is understandable as approximately 80% of the Ca$^{2+}$ forming the fetal skeleton at the end of gestation has been transported across the placenta from the mother.

In humans the fetus requires an average daily transfer of 200mg of Ca$^{2+}$, leading to a typical accretion of 21g by term (Kovacs et al. 2002). It is of particular interest to neonatologists as the precise measurement of mineral transport will aid in the supplementation of Ca$^{2+}$ to support fetal development in cases of premature birth or low bone mass (Abrams 2007). It has been estimated that the cytoplasmic concentration of Ca$^{2+}$ in the syncytiotrophoblast can be up to 1000 times higher than free circulating Ca$^{2+}$ in maternal circulation (Greer 1994). It is intriguing that this highly active trophoblast layer of the placenta, which undergoes relentless proliferation, differentiation and apoptosis, utilises the Ca$^{2+}$ ion as an intracellular messenger to mediate these cellular processes in a high Ca$^{2+}$ setting (Berridge et al. 2003). Further studies into placental regulation and homeostasis of calcium may increase our understanding of placental pathologies that affect healthy fetal development.

1.4.2.1 Calcium Transport and Homeostasis

Calcium transport by epithelial tissues is widely recognised across mammals to be of similar disposition. To begin with, apical Ca$^{2+}$ entry involves voltage gated epithelial Ca$^{2+}$ channels.
Next, $\text{Ca}^{2+}$ is ferried across the cell cytoplasm via a binding action before being deposited out of the cell via active transport on the BM (Bindels 1993). Following the identification of the $\text{Ca}^{2+}$ transporting process, more in depth studies have been conducted and the route of $\text{Ca}^{2+}$ through the placenta is postulated as follows: 1) diffusion into the trophoblast from maternal plasma down an electrochemical gradient through epithelial $\text{Ca}^{2+}$ channels of the transient receptor potential (TRP) gene family; 2) transfer across the trophoblast cytoplasm bound to the $\text{Ca}^{2+}$ binding protein calbindin-D$_{9K}$ and 3) active extrusion into the fetal compartment via the plasma membrane $\text{Ca}^{2+}$-ATPase localised to the fetal facing basal plasma membrane (figure 1.3.2). The three proteins characterised above are known as 1) Epithelium $\text{Ca}^{2+}$ channel (ECaC); 2) Calbindin-D$_{9K}$ and 3) $\text{Ca}^{2+}$-ATPase (PMCA) (Bond et al. 2008). $\text{Ca}^{2+}$ transport across the placenta is now considered a dynamic process as the placenta is thought to be able to adapt to meet fetal demand signals. Several factors, including the calcitropic hormone parathyroid hormone-related protein and the $\text{Ca}^{2+}$ sensing receptor (CaSR) on both the maternal and fetal side are partly responsible for the maintenance of $\text{Ca}^{2+}$ flux across the placenta.
Figure 1.4.2: Ca^{2+} transport through the syncytiotrophoblast. Showing diffusion into the trophoblast from maternal plasma down an electrochemical gradient through epithelial Ca^{2+} channels, transfer across the trophoblast cytoplasm bound to the calcium binding protein calbindin-D_{9K} (CaBP_{9K}) and active extrusion into the fetal compartment via the plasma membrane Ca^{2+}-ATPase localised to the fetal facing basal plasma membrane. Adapted from Slepchenko and Bronner which outlines calcium transport in the gut, the next section goes on to outline why this model is relevant to trophoblastic calcium transport also (Slepchenko and Bronner 2001).
1.4.2.2 Parathyroid hormone-related protein in calcium homeostasis

Parathyroid hormone-related protein (PTHrP), which is produced by the placenta (Tobias and Cooper 2004), was first identified as the humoral factor responsible for hypercalcemia of malignancy. Further investigation led to the identification of PTHrP in a range of fetal and adult tissues (Philbrick et al. 1996). PTHrP is a complex prohormone which has been shown to undergo post-translational cleavage by the action of prohormone convertases to create a family of peptides, each member of which has its own receptor. The availability of the PTHrP fetal knockout mouse (NL) have led to further elucidation of the role the protein plays in calcium regulation in the placenta. It has been shown that NL fetuses exhibit a significantly reduced fetal blood calcium concentration resulting in the suppression of the maternal-fetal Ca\(^{2+}\) gradient (Kovacs et al. 1996). The authors suggested that maternal-fetal Ca\(^{2+}\) flux was reliant upon fetal PTHrP regulation. Further investigation, however, has shown that the abolition of the PTHrP gene does not lead to a decrease total Ca\(^{2+}\) accretion in the fetus but merely decreases circulating Ca\(^{2+}\) levels. This indicates that although PTHrP may cause disruptions to Ca\(^{2+}\) transport and abolish the maternal-fetal concentration gradient, there is a compensatory mechanism that increases Ca\(^{2+}\) deposition in the skeleton of the NL fetus (Tucci et al. 1996). The role of PTHrP in maintaining Ca\(^{2+}\) homeostasis during gestation remains unclear. More recent work has shown, using in situ placental perfusion, that maternal-fetal Ca\(^{2+}\) transport across the placenta is increased despite reduction of Ca\(^{2+}\) in fetal circulation (Bond et al. 2008) thus indicating that placental Ca\(^{2+}\) transport is not reliant upon the presence of PTHrP.

1.4.2.3 Calcium sensing receptor

The calcium sensing receptor (CaSR) is expressed in numerous tissues including the parathyroid gland, bone, intestine, kidney, brain, skin, lung and heart (Aida et al. 1995; Bikle et al. 1996; Garrett et al. 1995; House et al. 1997; Malaisse et al. 1999; Riccardi et al. 1996; Ruat et al. 1995; Rutten et al. 1999). It is a G-coupled protein receptor with seven
transmembrane spanning regions which responds to extracellular \( \text{Ca}^{2+} \) concentration (Brown et al. 1995). Mutations in the gene which encodes human CaSR have been proven to cause a range of disorders associated with an altered response to \( \text{Ca}^{2+} \). These include familial hypocalciuric hypercalcemia, neonatal hyperparathyroidism and autosomal dominant hypocalcemia (Pollak et al. 1994; Pearce et al. 1996).

CaSR has been shown to influence both parathyroid and renal function (Hebert et al. 1997). In the parathyroid the CaSR responds to an increase in extracellular \( \text{Ca}^{2+} \) by inhibiting the production and secretion of parathyroid hormone (PTH) (Brown et al. 1995). A reduction in extracellular \( \text{Ca}^{2+} \) causes the CaSR to stimulate synthesis and secretion of PTH. PTH mediates skeletal resorption via osteoclast action and also stimulates increased renal reabsorption of \( \text{Ca}^{2+} \) (Kovacs et al. 1998). The presence of the parathyroid \( \text{Ca}^{2+} \) sensing receptor was confirmed in human placental cells by the use of reverse transcription PCR and northern blot analysis. However, the major transcript varied slightly to that expressed in human parathyroid cells (Bradbury et al. 1998).

The intricacies involved in maternal-fetal \( \text{Ca}^{2+} \) homeostasis advocated the exploration of the role of fetal CaSR. The use of a CaSR gene knockout mouse allowed for further research into fetal \( \text{Ca}^{2+} \) handling (Ho et al. 1995). These studies noted that CaSR located in the fetal parathyroid gland suppressed PTH secretion in response to the typically high circulating \( \text{Ca}^{2+} \) levels (Kovacs et al. 1998). The authors found that placental \( \text{Ca}^{2+} \) transfer was disrupted by alteration to the CaSR. Both \( \text{CaSR}^{+/+} \) and \( \text{CaSR}^{-/-} \) fetuses displayed an elevated blood \( \text{Ca}^{2+} \) level, however whilst the \( \text{CaSR}^{+/+} \) showed a modest reduction in relative \( \text{Ca}^{2+} \) transport across the placenta the \( \text{CaSR}^{-/-} \) fetus displayed a 60% reduction compared to wild type counterparts. The authors reasoned that due to the similar increase in circulating fetal \( \text{Ca}^{2+} \) between genotypes the alteration in the maternal-fetal \( \text{Ca}^{2+} \) gradient was not responsible for the changes in placental transfer of \( \text{Ca}^{2+} \) noted in \( \text{CaSR}^{-/-} \) (Kovacs et al. 1998). These findings also point to a compensatory mechanism that allows both genotypes to still achieve a hypercalcemic state, possibly due to a change in renal and/or skeletal calcium handling. It is thought the role of CaSR in the developing fetus is to suppress the
secretion of PTH in response to elevated circulating Ca\(^{2+}\) to maintain the divergence between fetal and maternal Ca\(^{2+}\) levels. It is believed that disruption of the CaSR gene disturbs fetal Ca\(^{2+}\) homeostasis by affecting PTHrP controlled Ca\(^{2+}\) transport (Kovacs et al. 1998).

1.4.2.4 TRPV6 Epithelial calcium channel

TRPV6 is the epithelial Ca\(^{2+}\) channel involved in the maternal-fetal transport of Ca\(^{2+}\). These Ca\(^{2+}\) specific channels are located on the apical microvillous membrane of the syncytiotrophoblast which is exposed to the maternal circulation. Ca\(^{2+}\) diffuses via an electrochemical gradient into the cytoplasmic compartment of the syncytium (Haché et al. 2011). These highly selective Ca\(^{2+}\) channels are part of the TRP super family and once the functionality of these proteins was elucidated they were labelled 'transient receptor potential vanilloid' (TRPV) channels (Harteneck et al. 2000). At present this TRPV subfamily comprises of isoforms TRPV5 and TRPV6. Previously, TRPV6 has been shown to act as the entry pathway in intestinal Ca\(^{2+}\) transport and is regulated by 1,25 dihydroxyvitamin D (Bianco et al. 2007). However TRPV6 was only recently identified in vivo as an important contributor in maternal-fetal Ca\(^{2+}\) transport. Although mRNA expression has been recorded in both human and murine placenta (Moreau et al. 2002) the exact physiological role has not yet been defined. Wild type mice display a 14-fold increase in placental TRPV6 mRNA over the last four days of gestation (term= E21). This coincides with an increase in other Ca\(^{2+}\) related transporter proteins that increase to meet fetal demand during fetal skeletal mineralisation. It was reported that TRPV6 knockout mice had significantly lower fetal blood calcium than wild type counterparts. The authors were able to deduce this hypocalcemia was potentially due to a decrease in maternal-fetal calcium transport (Suzuki et al. 2008). The TRPV6 knockout mouse also displayed a 40% decrease in radioactively labelled Ca\(^{2+}\) transport from the mother to the fetus when compared to their wild type counterparts (Suzuki et al. 2008). This measurement was taken in vivo and was the first evidence that
TRPV6 is involved in maternal-fetal Ca\textsuperscript{2+} transport. TRPV6 regulation has been studied during pregnancy in both mice and rats using anti-steroid hormone antagonist injections prior to maximal TRPV6 expression (Lee et al. 2009). The authors report that down-regulation of TRPV6 expression in the rat placenta after treatment with RU486 (a progesterone receptor antagonist) indicates that progesterone and its receptors may control placental TRPV6 transcription. In contrast to this the authors also report that TRPV6 expression in mice may be regulated by oestrogen after observing significant down regulation of TRPV6 after the introduction of ICI182,780 (an oestrogen receptor antagonist). This indicates that regardless of species, TRPV6 may be regulated by sex steroids (Lee et al. 2009).

1.4.2.5 Calbindin-D\textsubscript{9k} Calcium binding protein

Calbindin-D\textsubscript{9k}, with a molecular weight of 9-kDa is considered to be a reliable marker for epithelia Ca\textsuperscript{2+} transport (Morgan et al. 1997). It has two Ca\textsuperscript{2+}-binding domains and is part of an intracellular group of proteins which have high affinity for calcium (Christakos et al. 1989). These proteins undergo structural changes once binding has occurred. Unlike other Ca\textsuperscript{2+} transporting proteins however, each Ca\textsuperscript{2+} binding protein is encoded by a different gene and there is no association and no homology between separate genes (Persechini et al. 1989). Expression has been verified in numerous tissues including uterus, placenta, kidney, intestine, bone and pituitary glands (Choi and Jeung 2008). Placental transcripts of Calbindin-D\textsubscript{9k} were found in both the cytotrophoblasts and syncytiotrophoblast of human placenta at term. The syncytiotrophoblast had higher levels of expression than the cytotrophoblasts (Belkacemi et al. 2004). Over the last third of gestation there is a marked increase in the expression of Calbindin-D\textsubscript{9k} in both mouse and rat placenta (Mathieu et al. 1989;Glazier et al. 1992;Hamilton et al. 2000;An et al. 2004;Bond et al. 2008). This up regulation of Calbindin-D\textsubscript{9k} expression in the rat placenta was also linked to an increase in unidirectional maternal-fetal Ca\textsuperscript{2+} clearance over the same period. This led to speculation that Calbindin-D\textsubscript{9k} is rate limiting for the placental transport of Ca\textsuperscript{2+} in the rat (Glazier et al.}
In contrast to this, it is reported that the Calbindin-D$_{9k}$ knockout mouse was phenotypically normal and survived for up to a year postnatally suggesting there is a compensatory mechanism to cope with Calbindin-D$_{9k}$ deficiency (Lee et al. 2007). Moreover, further investigation into Calbindin-D$_{9k}$ null mice showed they were indistinguishable from wild-type counter parts in mice in both phenotype and serum Ca$^{2+}$ levels. The authors reason that Calbindin-D$_{9k}$ is not required in reproduction or Ca$^{2+}$ homeostasis (Kutuzova et al. 2006).

In the mouse placenta regulation and expression of Calbindin-D$_{9k}$ has been shown not to be dependent upon the vitamin-D receptor as has been shown in other tissue (Shamley et al. 1996). This led to further exploration and studies suggest that Calbindin-D$_{9k}$ mRNA in mouse placenta can be regulated by sex steroid hormones E2 and P4 and their receptors via complex pathways in the tissue. Furthermore placental control via sex steroid hormones versus known vitamin D control of Calbindin-D$_{9k}$ in the intestine allow for speculation that Ca$^{2+}$ binding is regulated in a tissue specific manner (An et al. 2004). It was also reported that placental Calbindin-D$_{9k}$ is marginally increased in expression in the PTHrP null fetus leading to the conclusion this particular isoform is regulated by PTHrP (Bond et al. 2008). The binding of Ca$^{2+}$ for transcellular transportation between apical and basal membrane comes with added benefits. The buffering action of binding, especially in the Ca$^{2+}$ rich trophoblast environment, allows for intracellular Ca$^{2+}$ to continue in its role as a signalling molecule independent from Ca$^{2+}$ transport (Koster et al. 1995).

It should been noted that Calbindin-D$_{9k}$ concentration in Ca$^{2+}$ transporting epithelia is often in the sub-millimolar range to complete its normal function (Feher 1983) and therefore accounts for the abnormally large increase seen in placental Calbindin-D$_{9k}$ in the last trimester of pregnancy. Interestingly studies have shown a remarkable co-localisation of Calbindin-D$_{9k}$ in any tissue expressing TRPV5/6 Ca$^{2+}$ channels and this could suggest a functional coexistence that is yet to be deliberated (Lambers et al. 2006). It is plausible that Ca$^{2+}$ binding is happening close to the channel entrance to maintain a Ca$^{2+}$ gradient and
thus influx. It must also be taken into consideration however that there may be a functional interaction between the two proteins that needs exploring (Shimura and Wasserman 1984).

1.4.2.6 Plasma membrane calcium ATPase

PMCA plays a key role in the active extrusion of intracellular Ca$^{2+}$ from the cell. In the placenta PMCA is located on the basal membrane of the syncytium. It was thought that PMCA was exclusive to the BM however it has been reported more recently that isoforms are found on the MVM. It also been reported that the PMCA localised to the MVM demonstrates higher activity and expression than that on the BM (Marín et al. 2008). In the BM, PMCA is the primary transporter mediating the extrusion of calcium out of the cell (Fisher et al. 1987; Strid et al. 2003). Four separate genes have been identified (PMCA1-4) which code PMCA (Zylińska et al. 2002). Isoforms 1 and 4 are expressed ubiquitously in all tissues, whereas isoforms 2 and 3 are found in more specialised cell types (Zylińska et al. 2002). To be functional, PMCA requires the presence of ATP. Due to its importance in the transcellular transport of Ca$^{2+}$, PMCA has been widely studied. In rat placenta a three-fold increase of PMCA gene expression was recorded which led to a 72-fold increase in total Ca$^{2+}$ across the placenta in the final days of gestation (Glazier et al. 1992).

1.4.3 Placental Calcium Transport and Pregnancy Complications

A study conducted in placentas from IUGR fetuses showed PMCA activity was increased by 48% whilst expression was reduced by 15% when compared to controls (Strid et al. 2003). Using rapid filtration techniques to quantify the transport activity of Ca$^{2+}$ into human syncytiotrophoblast basal plasma membrane vesicles, it was hypothesised that PMCA activity may be activated by IUGR. The ability of IUGR to alter the expression and activity of PMCA in the placenta supports suggestions that the placenta can respond to fetal demand growth signals through regulation and expression of specific placental transport systems (Constancia et al. 2005).
IUGR can be a consequence of preeclampsia, which is characterised by gestational hypertension, proteinuria, oedema and fetal syndromes. Although the exact pathogenesis of pre-eclampsia remains unclear it is considered to involve systemic failings (Sibai et al. 2005). Placentas of women with pre-eclampsia have been shown to have reduced expression of isoforms 1 and 4 of PMCA (Haché et al. 2011). Due to the afore mentioned dependency on the presence of ATP to remain functional, previous studies have shown that pre-eclamptic placental cells display lower ATP levels due to an alteration in a ATP-synthase gene (Hansson et al. 2006). In rat cells it has been shown that changes in PMCA expression correlate with its activity and ATP content (Nabekura et al. 2004). In term labour monitored for key pre-eclamptic marker soluble fms-like tyrosine kinase-1, it was recorded that TRPV6 and PMCA were highly expressed in both maternal and fetal sections of the placenta, the authors deduced this may be in reaction to impaired Ca$^{2+}$ metabolism across the syncytiotrophoblast to maintain fetal and maternal Ca$^{2+}$ balance (Yang et al. 2013).

1.5 **In Vivo and In Vitro Models of the Placenta**

The use of in vivo and in vitro models provides the ideal conditions in which scientific experiments can be conducted, although all results must be considered in the context of said conditions.

1.5.1 **In Vivo Rat Model of Pregnancy and Placental Function**

Both human and rat placenta are of a haemochorial nature which involves the modification of the maternal-fetal interface in order to accommodate nutrient transport to the developing fetus which is achieved in two stages. Firstly, nutrients from maternal circulation must be delivered to the placenta. Secondly, nutrients must be transferred from the placenta to the fetal compartment. To fulfil these requirements both species undergo extensive vascular remodelling of maternal spiral arteries as described in section 1.3 (Pijnenborg et al. 1981). Other similarities appear in the placental to fetal interface in which a syncytial barrier is
formed, the rat placenta utilises a hemotrichorial barrier which comprises of three trophoblastic layers whereas the human barrier is of hemomonochorial descent. Organisationally, there is variation in the trophoblastic make up. The human placenta utilises the syncytial trophoblastic layer on the maternal side with the cytotrophoblast layer localised to the fetal blood side of the cell. The rat placenta is different in that the cytotrophoblast layer is localised to the maternal side of the membrane and there are two syncytial trophoblast layers (Knipp et al. 1999). In both cases, however, the uterine-placental interface is also characterised by trophoblastic invasion of uterine tissue (Soares et al. 2012). In the human placenta the cytotrophoblast and syncytiotrophoblast cells are found in both villous and extra villous locations. In the rat, cells of the trophoblastic lineage are very zone specific - trophoblast giant cells are located in the junctional zone and the labyrinthine whereas the spongiotrophoblasts and the syncytial trophoblast cells are located only within the junctional zone and labyrinth zone respectively (Knipp et al. 1999).

In human gestation the placenta continually gets bigger as gestation progresses. Although the rate of growth slows in the last few weeks, villous tree development and branching, increase in exchange surface areas and DNA synthesis have all been found up until 42 weeks of gestation (Fox 1997). The rodent placenta, however, reaches it largest size at mid to late gestation and begins to decrease towards term as the rapid growth of the fetus at term utilises placental energy and nutrient stores (Coan et al. 2004).

Although transport of calcium and amino acids across the rodent and human placenta are well characterised (Glazier et al. 1992; Dilworth et al. 2010; Bond et al. 2008) there are notable differences in the rodent. The intra placental yolk sac is found exclusively in the rodent placenta and has previously been shown to express higher levels of calbindin-D$_{9k}$ than surrounding trophoblasts (Bruns et al. 1985). Subsequently, the increased expression of calcium transporting proteins recorded in the rodent placenta towards the later stages of gestation is mirrored in the intra placental yolk sac which leads to a larger increase in calbindin-D$_{9k}$ than recorded in trophoblasts (Mathieu et al. 1989). Further to this the intra placental yolk sac has also been shown to express PMCA (Borke et al. 1989).
The rat is a well characterised model of human pregnancy with similarities in placental structure and function to those seen in the human placenta. However, as with any model, differences will ultimately cause limitations when translating data interpretation between the rat and human.

1.5.2 In Vitro Cellular Model of the Placenta: BeWo

BeWo are a human cell line that originated from a choriocarcinoma which was first isolated over 40 years ago (Pattillo and Gey 1968). Cells were initially separated and transferred to the cheek pouch of a hamster before being placed in co-culture with human decidu al explants (Pattillo and Gey 1968). They have since been maintained as a continuous immortal cell line for a placental in vitro model. BeWo cells retain the endocrine function of trophoblastic cells in that they secrete hCG and hPL. These hormones used to be the criteria for identification as a trophoblast cell type; however it is now clear that other non-trophoblastic cell lines also produce hCG (Duffy 2001). The new standard relies on the expression of cytokeratin-7, HLAG or CD9, markers which correspond to trophoblastic and extravillous cell types, of which BeWo express two and thus this cell line is a mix population of cells (Sullivan 2004).

Isolated human placental cell lines retain many morphological aspects of in vivo trophoblast cells and BeWo are no exception. They resemble both undifferentiated cytotrophoblasts and syncytialised trophoblast cells (Wolfe 2006). Continuous cell culture models are often used in experiments as they infer benefits that in vivo work or isolated primary cells cannot. BeWo cells are used in trans-cellular studies over isolated placental fragments or primary cells because they do not spontaneously syncytialise and leave gaps on the growing surface (Jones et al. 2006a). BeWo cells are utilised in a range of placental function experiments including, but not limited to; effects of xenobiotics, endocrine function, trans-placental transfer, cellular efflux and transporter elucidation experiments (Myllynen and Vähäkangas 2013). Another benefit of using a cell-line is that you are able to conduct experiments on single cell types without having other placental cell types affecting results.
Choriocarcinoma cell lines also have disadvantages; it is uncertain what effect that other placental cell types can have on the function of trophoblasts and by removing them from a whole placental environment you may be altering the behaviour that would be seen in vivo. Furthermore, BeWo lack the function of some nutrient transporters and some argue it is difficult to extrapolate data obtained using cell lines into an in vivo context (Kitano et al. 2004). Further complications arise when the longevity of the cell line comes into consideration; different laboratories report intra-BeWo variation with regards to fusion rates and syncytialisation. It has previously been reported that trophoblast cell lines in culture can undergo phenotypic drift when kept in sub optimal conditions thus leading to a change of characteristics (Orendi et al. 2011).

1.6 CIRCADIAN RHYTHMS

Physiological and behavioural processes have adapted to be cyclical in nature to allow organisms to more effectively cope with predictable daily fluctuations in the external environment. Oscillations in function are driven by endogenous biological clocks which are found system wide and co-ordinate a variety of physiological processes to align with behaviour; these include cell proliferation and metabolism (Asher and Schibler 2011). The advantage of maintaining a molecular clock is still disputed, however it has been shown that under selective pressure simple organisms garner a fitness advantage with functioning and suitable circadian machinery.

It has been shown that cyanobacteria outgrow competitors in an environment that is specific to the period length of their circadian oscillations (Woelfle et al. 2004). In organisms of a more complex nature it is now widely accepted that disruptions to circadian function and gene mutations can lead to infertility and cancer (Fu et al. 2002). It is agreed that the hypothalamus is the home of the mammals circadian timing system (Rusak and Zucker 1979), named the suprachiasmatic nucleus (SCN). Work conducted specifically on the SCN has shown that isolation of a single neuron does not disrupt its ability to generate a circadian oscillation (Michel et al. 1993). This lead to the hypothesis that rather than being responsible
for the generation of circadian rhythms organism wide, the SCN acts to regulate, synchronize and orchestrate circadian rhythms via peripheral clocks located in almost every tissue including the placenta (Wharfe et al. 2011). Light/dark cycles entrain the SCN via light receptive ganglion cells located in the retina (Lowrey and Takahashi 2000) and in turn peripheral oscillations are synchronised by endocrine and autonomic action governed by the SCN (Hastings et al. 2007). Work conducted using an implanted SCN in porous plastic was able to reverse the abolition of circadian oscillations in animals which had lesions on the SCN. This was the first evidence to suggest that circadian cues were primarily hormonal rather than electrical as was previously thought (Silver et al. 1996). Further work conducted using immortalised cell lines in culture, demonstrated the ability of the serum shocking technique to synchronise the cells circadian machinery in 35 year old rat fibroblasts. Serum shocking is postulated to work in 1 of 2 ways, firstly, un-synchronised cell circadian rhythms are re-synchronised by the addition of the high concentration serum media, or secondly; the serum shock technique works to kick start dormant rhythms in isolated cells. This work was the beginning of a range of experiments which demonstrate that detailed circadian machinery is in fact ubiquitous to most tissues of the mammalian body (Balsalobre et al. 1998).

Synchronisation is thought to happen in a hierarchical manner. External timing cues, predominantly light, entrain the central timing machinery or pacemaker. The SCN contains independently cycling neurons that subsequently project the entrained rhythm into the autonomous peripheral tissue's circadian machinery (Kowalska and Brown 2007). Despite increased understanding into the workings of the molecular clock, the way in which it influences physiological behaviour and gene expression remains unknown. Current research suggests the SCN communicates with peripheral circadian machinery through several different pathways and mechanisms. Peripheral circadian machinery can directly control gene expression via transcription factor cascades.
1.6.1 Clock Genes

It is now widely agreed that peripheral clocks control gene expression and physiology rather than the master clock in the SCN (Kowalska and Brown 2007). These central and peripheral circadian systems are made up of a network of ‘clock’ genes, including CLOCK, BMAL1, Per1-3, Cry1 and 2. These genes form complicated feedback loops using transcriptional and translational processes to drive oscillating rhythmic expression of downstream genes, thus leading to rhythmic patterns in physiological processes.

Elucidation of the workings of clock machinery was undertaken after the mapping of circadian genes using N-ethyl-N-nitrosourea (ENU) mutagenesis (Vitaterna et al. 1994). CLOCK and BMAL1 form the positive limb of the transcriptional feedback loop. CLOCK and BMAL1 undergo transcription and translation and the proteins pool in the cell cytoplasm. CLOCK proteins undergo post translational modifications to increase stability and cellular localisations to maintain accuracy of the circadian machinery. It also acts to delay feedback inhibition by the negative limb by delaying transcriptional activation (Chen et al. 2009; Gallego and Virshup 2007). CLOCK and BMAL1 then interact to form a heterodimer in the cell cytoplasm. Once the CLOCK/BMAL1 dimer is constructed the formation moves back into the nucleus via BMAL1 shuttling.

Studies have shown that BMAL1 contains a functional nuclear localisation signal in the N-terminal, this means BMAL1 is crucial to a functioning circadian oscillation as it allows the CLOCK/BMAL1 complex to aggregate to the cell nucleus (Kwon et al. 2006). It is the shuttling of the CLOCK/BMAL1 protein heterodimer which is key in driving circadian oscillations. The post translational events, including the stabilisation of CLOCK and the shuttling of BMAL1 are key and have been described as the activators of the mammalian clock system (Kondratov et al. 2003).

Once in the nucleus the CLOCK/BMAL1 heterodimer interacts with an E-Box enhancer element - a DNA sequence that can act as a protein binding site (Hao et al. 1997) - on the promoters of circadian controlled genes to drive the expression of all other core clock genes.
Include Cry and Per. These proteins act as the negative agents in the feedback loop. At this point the expression of Per and Cry proteins is increased and they interact in a similar fashion and translocate into the nucleus to inhibit the activity of CLOCK and BMAL leading to degradation of the formed heterodimers in the nucleus. Consequently, there is a decrease in stimulation of core clock gene production (Hirota et al. 2002). After the inhibition of the CLOCK/BMAL1 heterodimer via the action of Cry and Per, circadian gene expression is no longer driven by the binding of CLOCK/BMAL1 to the E-Box enhancer elements on the genes. As such, Cry and Per are no longer there to inhibit CLOCK/BMAL1 action and thus the transcription translation oscillating loop is formed. This has become known as the transcription translation oscillating loop model (TTO).

1.6.2 TTO AND CORE CLOCK GENES

Other proteins have been identified as accessory proteins. These include; D-Box binding proteins, the retinoid-related orphan receptor and the nuclear receptor subfamily 1. These accessory proteins are all thought to support and modulate the activity of the TTO loop (Richards and Gumz 2012).

Although the same basic components in the central and peripheral circadian systems are both capable of generating oscillations, it has been noted that mutations in circadian genes have exaggerated effects within peripheral tissues. It has been shown that deletion of the Per1 gene decreases the period of a circadian oscillation by approximately an hour yet in isolated fibroblasts the same deletion causes a four hour discrepancy (Brown et al. 2005). In contrast to this, it has been recently reported that the abolition of CLOCK does not disrupt circadian behaviour. It is postulated that other proteins are able to substitute in order to retain function. Some peripheral tissues may not express these substitute proteins leading to a bigger discrepancy in peripheral circadian oscillations compared to the SCN (Debruyne et al. 2006). In addition to this, it has been reported that there is greater neuronal coupling in the SCN compared to peripheral counterparts resulting in better intracellular communication and thus better adherence to the circadian clock (Welsh et al. 2004).
1.6.3. DIETARY CUES OF THE CIRCADIAN CLOCK

How diet and feeding cues can influence the circadian clock has come under close scrutiny in recent years. The synchronicity of the CLOCK and BMAL1 regulation has been shown to be directly involved with glucose homeostasis. The inactivation of CLOCK and BMAL1 leads to the daily variation in the concentration of glucose and triglycerides. Gluconeogenesis is reported to be down regulated in CLOCK mutants, whereas it is totally abolished by deletion of the BMAL1 gene (Rudic et al. 2004). It is possible that CLOCK or BMAL1 may also work independently of the circadian clock mechanism to exert effects elsewhere. This must be considered when using knockout mice models to elucidate the function of the circadian clock (Rudic et al. 2004). Investigation into how diet can influence the circadian clock has found that a high fat diet is able to manipulate circadian variation by changing carbohydrate metabolism (Kohsaka et al. 2007).

Maintenance of blood glucose levels has always been critical to mammalian survival and it has been demonstrated that CLOCK and BMAL1 contribute to recovery from the hypoglycaemic response to insulin. Changes in feeding patterns have also been noted to shift the circadian gene expression in peripheral clocks. It was shown that alterations to feeding patterns brought about a change in circadian gene expression in the liver which was not seen in the master clock in the SCN (Damiola et al. 2000). Glucose was also able to induce a rhythmic oscillation of gene expression in isolated fibroblasts (Hirota et al. 2002). More recent studies have also examined the role the circadian clock plays in glucose homeostasis; investigators reported a delayed circadian oscillation in glucose metabolism and insulin signalling in circadian mutants (Marcheva et al. 2010). Pancreatic islets were shown to express peripheral circadian machinery and CLOCK disruptions led to transcriptome wide changes in growth and survival (Marcheva et al. 2010). As with all homeostatic processes the circadian machinery is not the only mechanism exerting its effects upon the regulation of glucose and it works in conjunction with other processes such as steroids, epinephrine and glucagon (Hochgeschwender et al. 2003).
Although recent studies have highlighted how circadian oscillations can impact behaviour and cell physiology, it is still hard to envisage the potential of circadian study to characterise human disorders. Maintenance of a subject under control conditions is costly and even though some peripheral tissues are widely available; namely blood, hair and skin, the nature of peripheral oscillations and circadian machinery results in the tissues not being functional for studies of pathologies that do not take place in these tissues.

1.6.4. **Circadian Activity in the Placenta**

Rhythmic placental activity has previously been observed in the rat when analysing fetal growth rate. It was noted that two-thirds of total daily dry and wet fetal weight was gained during the active (dark) phase of the rats daily routine (Barr 1973). Although this observation was in part due to the maternal circadian behaviour of the mother, it does suggest the placenta may display rhythmic activity. Recent reports indicate that disruption to normal light-dark cycles can reduce fetal growth in the rat by up to 24% at term (Gozeri et al. 2008). These altered light schedules that the rat was exposed to resulted in abnormal placental histology. This included an increase in placental oedema, fibrin accumulation and leukocyte infiltration. A somewhat more tenuous link between altered circadian behaviour and adverse fetal outcome in humans has been made when looking at both regular air travel and shift work during pregnancy. Low birth weight and pre-term birth both have both been associated with these activities (Mahoney 2010).

In the extravillous trophoblast cell line HTR-8/SVneo oscillatory expression of Per2 and Vegf occurred after a serum shock procedure which has been shown to induce cyclic expression of clock genes in other tissues (Balsalobre et al. 1998; Frigato et al. 2009). However, there are no reports of cyclic circadian activity in the intact human placenta. In rodent models, circadian oscillations have been recorded in the placenta for several clock genes. The expression of said genes was noted to be very zone specific. The labyrinthine zone of the placenta expressed higher levels of CLOCK, whereas the junctional zone was recorded to have increased BMAL1 levels (Wharfe et al. 2011). The relative amplitude recorded during
the cyclic activity was very low in the mouse and in the rat the cyclic activity was very phase dependent however all clock genes peaked in the dark phase (Wharfe et al. 2011). This is at odds with other tissues that display circadian variation where the afore mentioned feedback loops drive cyclic expression (Hastings et al. 2007).

Despite clear evidence that the placenta does express clock genes, the impact they have on placental function is still unclear. There is a need for specific studies that look into the effect that circadian machinery has on the placenta and pregnancy outcome.

1.7 SUMMARY AND AIMS

A religious period of IF can sometimes coincide with pregnancy. Although women are exempt from the fasting period many choose to participate on social grounds. It was noted that a period of fasting during specific trimesters of gestation lead to a reduced placental size at term. The placenta however was able to maintain fetal growth so there was no disparity between fetal sizes at term. To this end the placenta must be able to meet fetal demand signals via the transfer of nutrients from the maternal blood to the fetal compartment. Healthy structural developments of the nutrient transporting zones in the placenta are key to this and it is the syncytiotrophoblast that is primarily responsible for nutrient transfer. Both calcium and amino acid transport must be maintained from the beginning of gestation until term at the normal rate as both have previously been linked to altered fetal outcome. Another aspect of IF that challenges placenta function is the altered dietary cues and sleeping pattern which arise from the fasting period. Placental circadian regulation has not been widely explored especially with regards to the nutrient transporting capabilities of the syncytiotrophoblast.

A review of current literature has highlighted a disparity between the amount of women participating in IF during gestation and our understanding of the impact it may have on both normal placental development and fetal outcome. The aims of this study are to establish an in vivo IF model in the rat and characterise changes in protein expression of key nutrient transporting proteins for calcium and amino acids, and the regulatory proteins involved with
the circadian clock. The report then goes on to investigate the localisation of circadian
machinery in the main nutrient transporting zone of the human placenta using the human
choriocarcinoma cell line BeWo and looks to establish a link between circadian oscillations
and the protein expression of key nutrient transporters.

It is important that we document changes that could occur in placental development/function
due to a period of IF. The information will allow women to make informed decisions with
regards to the development of the fetus or it may lead to possible dietary supplementation to
assist fetal development during the IF period.
CHAPTER 2: MATERIALS AND METHODS
2.1 MATERIALS

All reagents were purchased from Sigma-Aldrich, Dorset, UK unless specified otherwise. Animal work described in sections 2.2.1 was conducted by Dr N. Ashton and colleagues at the University of Manchester, UK.

2.2 INTERMITTENT FASTING ANIMAL STUDY

2.2.1 ANIMALS AND HUSBANDRY

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. Wistar female rats (sourced from Charles River United Kingdom (CRUK)) were housed in Biological Services Unit (Stopford Building, Manchester) in a 12:12 hour light-dark cycle between 21-23°C with unlimited water supply. Females were mated with male studs with pregnancy day E0 defined as the presence of copulation plug. Intermittently fasted rats (n=9) had access to food denied from 5pm-9am daily throughout gestation whereas control counterparts (n=11) had unlimited access to standard pellet chow (Beekay Rat and Mouse Diet; Bantin and Kingman; Hull, UK). Dams were culled at term (E21) by Schedule 1 techniques and placentas were harvested from the uterine horn. One randomly assigned placenta from each litter was individually snap frozen in liquid nitrogen and stored at -80°C until required. Food intake and maternal weight was recorded daily from gestation until term. At E21 both fetal and placental weight was recorded.

2.2.2 HOMOGENISATION OF PLACENTAL TISSUE

Individual placentas were placed into a 55ml dounce homogenizer (Fisher Scientific, Loughborough, UK) with ice cold 1% PIC-RIPA buffer (homogenisation buffer). Samples were treated with 1ml homogenisation buffer per 0.1g of tissue. Tissue samples were homogenised on ice and prepared samples were transferred to 15ml falcon tubes (Fisher) and centrifuged at 2500g (MSE Mistral 3000i Centrifuge, London, UK) at 4°C for 5 minutes. The post nuclear supernatant (PNS) was drained; half was frozen at -80°C in 1.5ml Micro-
centrifuge tubes (Fisher). To obtain the cytoplasmic fraction the remaining PNS was transferred to Beckman Ultracentrifugation tubes and spun at 100,000g for 1 hour at 4°C (Beckman-Coulter Optima L-100K Ultracentrifuge, High Wycombe, UK). The PNS was discarded and the pellet re-suspended in 100µl of homogenisation buffer to acquire the membrane fraction. Samples were then stored at -80°C in 0.5ml micro-centrifuge tubes (Fisher) until used.

### 2.2.3 Protein Quantification

Protein concentration of homogenised rat placental samples was calculated using the Bradford’s micro plate assay method (Bradford 1976). Known BSA protein concentrations (0-1000µg/ml) were used to construct a standard curve off which placental samples could be read to determine protein concentration. Placental homogenates were diluted 1 in 40 before standards and samples were pipetted onto a 96 well plate (Costar Cambridge, MA USA). 200µl of Bradford’s reagent (at room temperature) was mixed into all wells before the optical density (OD) was measured at 595nm using a FLUOstar OPTIMA plate reader (BMG Labtech GmBH, Offenburg, Germany). MARS Analysis software was used to determine protein concentrations.

<table>
<thead>
<tr>
<th>Protein Concentration (µg/ml)</th>
<th>BSA mg/ml (µl)</th>
<th>DH2O (µl)</th>
<th>Bradford’s Reagent (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
<td>200</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>8</td>
<td>200</td>
</tr>
<tr>
<td>400</td>
<td>4</td>
<td>6</td>
<td>200</td>
</tr>
<tr>
<td>600</td>
<td>6</td>
<td>4</td>
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</tr>
<tr>
<td>800</td>
<td>8</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>1000</td>
<td>10</td>
<td>0</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 2.2: BSA concentrations used to construct a standard curve.
2.2.4 Gel Electrophoresis

Protein extracted from rat placental samples (20-50 µg) was loaded on to a polyacrylamide bis tris gel (4-12% or 12%; Invitrogen Ltd., Paisley, UK) after the addition of 4x sample buffer (Invitrogen Ltd.) and DH2O. In some cases samples were heated at 70°C for 10 minutes and/or reduced via the addition of 500 mM dithiothreitol reducing agent (Invitrogen Ltd.) (table 2.3). 10µl of pre-stained protein standard (3.5-260kDa; Invitrogen Ltd.) was also loaded into a single well on each gel. Gels were then subjected to vertical electrophoresis using a X-cell II system (Invitrogen Ltd.) in 1xMES running buffer (Invitrogen Ltd.) at 200V and 400mA until sufficient protein migration was observed.

2.2.5 Electro-Blotting

Separated proteins were blotted on to polyvinylidene fluoride (PVDF) membrane (Millipore, Watford, UK) using the X-cell II system. The gel-membrane assembly was constructed as shown in figure 2.2 using Whatmans grade 4 filter paper. Proteins were transferred for 90 minutes at 30V and 400mA in cold transfer buffer (1.45g Tris base, 7.2g glycine, 200ml methanol and 800ml dH2O). The blotting process was conducted on ice.

2.2.6 Probing the Membrane

Membranes were blocked using specific concentrations of non-fat milk powder (Sainsbury’s, UK) and Tween-20 diluted in either PBS or TBS (Table 2.3; 1.21g Tris, 8.18g sodium chloride, 1l DH2O, pH7.4) for 30 mins at 4°C. Membranes were then subjected to overnight incubation with primary antibodies at 4°C with gentle agitation. Appropriate secondary antibody washing was then conducted using either Goat Anti-Rabbit or Goat Anti-Mouse conjugates at a dilution of 1:5000 in the relevant antibody buffer shown in table 2.4. All membranes were probed with β-Actin to ensure equal protein loading.
Figure 2.2: Gel/Membrane complex used in the Western blot procedure.
2.3 CULTURING OF FIRST TRIMESTER CHORIOCARCINOMA CELL LINE, BEWO

2.3.1 BEWO

The BeWo (ATCC® CCL-98™) (passages 2-9) cell line was grown in 10, 17, 20, 30 or 45mmol/l glucose Dulbecco’s Modified Eagles Media (DMEM). Medium was supplemented with 10% fetal bovine serum (FBS) and 2% Penicillin-Streptomycin. BeWo cells were cultured in media containing 17mmol/l glucose unless it is specifically stated in the methodologies otherwise. A personal communication (Dr Westwood M, Dr. Forbes K and Prof. Aplin JD; Maternal and Fetal Health Centre, University of Manchester) indicates placental trophoblast cells are commonly cultured in a media with a supra-physiological concentration of glucose measuring 17mmol/l. This is possibly due to the high glucose consumption that occurs in BeWo. The cells were incubated at 37˚C with 5% carbon dioxide (CO₂)/air and 100% humidity and the culture media was replaced every 48 hours.

2.3.2 FREEZING AND THAWING OF CHORIOCARCINOMA BEWO CELLS

BeWo cells were given fresh media 24 hours prior to freezing. Confluent cells were trypsinised and incubated at 37˚C for 5 minutes to detach cells from the bottom of the culture flask. The 0.05% trypsin/EDTA solution was deactivated by the addition of fresh culture media. The resultant cell suspension was centrifuged (1000g for 5 min) and the supernatant was removed. The pellet was re-suspended in cold cell culture media (supplemented with 10% dimethyl sulphoxide (DMSO) and 20% FBS) at a density of 3x10⁶cells per cryo-vial. Cryo-vials were kept in a ‘Mr. Frosty’ freezing container at -80˚C overnight before being transferred to a liquid nitrogen store at (-176˚C) until required. The ‘Mr. Frosty’ helps avoid the formation of ice crystals via the use of isopropanol, which cools at a rate of 1˚C per minute, thus reducing cell lysis.

Rapid thawing of cells was required to avoid damage because DMSO becomes toxic to cells when above 4˚C. Cryo-vials were quickly thawed using a 37˚C water bath, cold cell culture media was added, the cell suspensions were centrifuged (1000g for 5 min) and the
supernatant was removed. The pellet was re-suspended in fresh media and transferred to a 25cm² culture flask. Once confluent, the entire cell population was transferred to a 75cm² flask and normal passage procedure was followed thereafter.

2.3.3 CELL PASSAGE

BeWo cells were sub-cultured to 80% confluence or above in 75 cm² culture flasks and once confluent they were transferred to a new culture flask or plate. Spent media was removed and cells were washed 3 times with Dulbecco’s PBS (−Mg²⁺ and −Ca²⁺) to remove media and left over serum. Once washed, cells were subjected to 0.05% trypsin/EDTA solution at 37°C for 5 minutes. Trypsin was then deactivated with the addition of fresh culture media (10% FBS, 2% pen-strep). The resultant cell suspension was subsequently dispersed via repeat gentle pipetting, split into the desired number of aliquots and transferred to new culture flasks or plates with fresh media.

2.3.4 SEEDING DENSITIES

For experimental work using BeWo cells, 75cm² and 25cm² flasks were seeded at 0.1x10⁶ cells per ml, whereas 96 well plates were seeded at 0.01x10⁶ cells per ml.

2.3.5 DETERMINATION OF CELL NUMBER

Following growth of cells in 75cm² culture flasks, sub confluent cells were harvested using 0.05% trypsin/EDTA and cells were manually counted using a haemocytometer and phase shift microscopy. Cells were counted using the haemocytometer via a glass gridded slide consisting of two chambers, each divided into nine 1x1mm squares. A glass cover slip was placed over the grid. Cell suspensions were loaded into the described chambers via capillary action. The cells occupy the space above the grid in a volume of 0.1µl for each 1 x 1mm square and were counted manually and the average cell number was calculated.
2.3.6 Viability Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay utilises cellular enzymes that reduce MTT to its insoluble configuration formazan, which is purple in colour. 2g of MTT was added to 400ml ultra-pure H₂O (5g/L), the subsequent solution was gravity filtered and then subjected to sterile filtration via syringe action using 0.22µm pore size. The relevant fresh cell culture media was made up to 10% MTT. 200µl of the media/MTT solution was added to each well on 96 well plates. The plates were then incubated for 4 hours at 37°C, with 5% carbon dioxide (CO₂) and constant humidity. Once complete media/MTT solution was drained and 150µl of DMSO was added to each well. Absorbance was then read using a Tecan Infinite® F50 Robotic plate reader (Tecan Group Ltd., Switzerland) with UV at 540nm.

2.3.7 Glucose Concentration on BeWo

All experiments were conducted at passage 9, prior to that BeWo cells were cultured in the relevant media; 10, 20 30 or 45mmol/l glucose, seeded as described in 2.3.4, in 75cm² culture flasks. Cells were incubated at 37°C, with 5% CO₂/air and constant humidity until confluence was achieved. Once confluent whole cell lysate and nuclei fractions were isolated as described below. This experiment was undertaken to ascertain whether BeWo cells contained functioning key circadian regulators. These particular glucose concentrations were used based upon studies previously conducted and their effects upon BeWo growth and confluence.

2.3.8 Serum Shock Procedure

Cells were seeded as described in 2.3.4 in 25cm² tissue culture flasks and grown until confluence was achieved. At this stage cells were washed twice with PBS before all liquid was carefully aspirated. 4ml of serum shocking media, 17mmol/l glucose DMEM media comprising of 50% horse serum 2% penicillin-streptomycin, was added to each flask. BeWo
cells were incubated 37°C with 5% carbon dioxide and constant 100% humidity for 2 hours in the ‘serum shock’ media. Once this 2 hour period was complete media was aspirated off and cells washed twice with PBS. Once PBS washes were complete this was considered as time 0 (T0). 4ml of serum free 17mmol/l glucose DMEM 2% penicillin-streptomycin was then added to each flask and cells were harvested every 6 hours for a period of 48 hours. Control flasks were subjected to the same wash routine, however they were always incubated with 10% fetal bovine serum (FBS) and 2% Penicillin-Streptomycin.

2.3.9 Collection of Whole Cell Lysate

Media was carefully aspirated from flasks and cells were washed twice with ice cold PBS. This was then drained and ice cold 1% PIC-RIPA buffer was added to flasks. Cells were detached from the bottom of culture flasks via cell scraping (Sarstedt, UK). The resulting cell suspension was transferred to micro centrifuge tubes and spun at 10,000g for 10 minutes at 4°C. The supernatant was carefully removed and stored as whole cell lysate at -80°C until required for further analysis.

2.3.10 Nuclei Isolation

Nuclei isolation was conducted using Sigma’s EZ prep kit. Confluent cells in either 25cm² or 75cm² tissue culture flasks were placed on ice and washed with 10ml ice cold PBS before the careful aspiration of the wash solution. 4ml of ice cold Nuclei EZ lysis buffer was added to the flasks. Cells were harvested and lysed via cell scraping and the entire cell lysate was transferred to a separate 15ml centrifuge tube. The solution was vortexed for 10 seconds and set on ice for 5 minutes. Nuclei were collected from the solution by gentle centrifugation, 500g for 5 minutes at 4°C, and the pellet was kept on ice. The supernatant was carefully aspirated off and saved as the cytoplasmic fraction for later use. The nuclei pellet was re-suspended in a further 4ml of Nuclei EZ lysis buffer as follows; firstly 0.5ml was added and the solution was briefly vortexed for 10 seconds. Secondly, the remaining 3.5ml of Nuclei EZ lysis buffer was added and the solution was vortex mixed for a further 10 seconds before the
entire solution was set on ice for 5 minutes. Nuclei were once again collected via gentle centrifugation at 500g for 5 minutes at 4°C and the supernatant was carefully aspirated off. The nuclei pellet was re-suspended in 200µl of cold Nuclei EZ storage buffer with clumps of nuclei being broken up via slow trituration with a micropipette. The final nuclei suspension buffer was transferred to micro centrifuge tubes for storage and further analysis.

2.3.11 TRYPSAN BLUE NUCLEI QUALITY ASSAY

The purity of the final nuclei yield was determined using a trypan blue assay and visual microscopic inspection. As the Nuclei EZ storage buffer used in section 2.3.10 contains glycerol, nuclei would swell if diluted in an aqueous solution therefore a 30% glycerol solution was used. 3ml of glycerol was diluted in 7ml of DH$_2$O and trypan blue 0.4% was added at a ratio of 4:1 glycerol solution-trypan blue. Nuclei in storage buffer were then added again at a ratio of 4:1 trypan blue glycerol solution-nuclei in storage buffer solution. This was then pipetted on to a haemocytometer and inspected using microscopy (EVOSXL Core, Life technologies, UK).

2.3.12 NUCLEI SONICATION

Nuclei sonication was used to disrupt DNA and nuclear envelopes in order to make the solution less viscous. The nuclei solution was sonicated at 70% amplitude for a total time of 2 minutes, 10 second pulse with 5 seconds off on ice.

2.3.13 WESTERN BLOTTING OF BEWO WHOLE CELL LYSATE AND NUCLEI FRACTIONS

BeWo whole cell lysates were subjected to the same procedures described between 2.2.3 and 2.2.6 to identify and quantify protein expression.
2.4 PROTEIN DETECTION

Membranes were subjected to extensive antibody washes after protein separation described in section 2.2.4-2.2.6. Table 2.3 and 2.4 outline optimised conditions for each Western blot.

2.4.3 PROTEIN VISUALISATION AND QUANTIFICATION

Protein detection and quantification was conducted using LI-COR’s Odyssey imaging machine and software (LI-COR Biosciences, Lincoln, Nebraska, USA).

2.4.1 OPTIMISED GEL RUNNING CONDITIONS

<table>
<thead>
<tr>
<th>Protein of Interest</th>
<th>Rat Placenta</th>
<th>BeWo</th>
<th>Fraction</th>
<th>Total Protein Loaded (µg)</th>
<th>Gel %</th>
<th>Heat Denatured</th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV6</td>
<td>✓</td>
<td>✗</td>
<td>Membrane</td>
<td>20</td>
<td>4-12</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>Calbindin-D9k</td>
<td>✓</td>
<td>✗</td>
<td>Cytoplasmic</td>
<td>50</td>
<td>12</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>PMCA</td>
<td>✓</td>
<td>✗</td>
<td>Membrane</td>
<td>50</td>
<td>4-12</td>
<td>✗</td>
<td>✗</td>
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<tr>
<td>SNAT 2</td>
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<td>4-12</td>
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<td>✗</td>
</tr>
<tr>
<td>CLOCK</td>
<td>✓</td>
<td>✓</td>
<td>Cytoplasmic/Nuclear</td>
<td>50</td>
<td>4-12</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>BMAL1</td>
<td>✗</td>
<td>✓</td>
<td>Cytoplasmic/Nuclear</td>
<td>20</td>
<td>4-12</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>IGF-II</td>
<td>✓</td>
<td>✗</td>
<td>Membrane</td>
<td>40</td>
<td>12</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

Table 2.3: Running conditions for different proteins of interest in rat placental samples
### 2.4.2 Optimised Antigen Conditions

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Host</th>
<th>Blocking Buffer</th>
<th>Antibody Buffer</th>
<th>Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV6</td>
<td>Santa-Cruz Biotechnology Inc</td>
<td>Rabbit</td>
<td>2% Milk Powder, TBST 0.1%</td>
<td>TBST 0.1%</td>
<td>1:1000</td>
</tr>
<tr>
<td>Calbindin-D9k</td>
<td>SWANT</td>
<td>Rabbit</td>
<td>3% Milk Powder, TBST 0.05%</td>
<td>3% Milk Powder, TBST 0.05%</td>
<td>1:1000</td>
</tr>
<tr>
<td>PMCA</td>
<td>Gene-Tex</td>
<td>Mouse</td>
<td>2% Milk Powder, PBS</td>
<td>PBST 0.05%</td>
<td>1:1000</td>
</tr>
<tr>
<td>SNAT 2</td>
<td>Santa-Cruz Biotechnology Inc</td>
<td>Mouse</td>
<td>2% Milk Powder, PBST 0.1%</td>
<td>1% Milk Powder, PBST 0.1%</td>
<td>1:1000</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Ab-Cam</td>
<td>Rabbit</td>
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<td>TBST 0.1%</td>
<td>1:10,000</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Millipore</td>
<td>Mouse</td>
<td>2% Milk Powder, PBST 0.005%</td>
<td>PBST 0.005%</td>
<td>1:200</td>
</tr>
<tr>
<td>BMAL1</td>
<td>Ab-Cam</td>
<td>Mouse</td>
<td>2% Milk Powder, TBST 0.1%</td>
<td>TBST 0.1%</td>
<td>1:100</td>
</tr>
<tr>
<td>Beta-Actin</td>
<td>Ab-Cam</td>
<td>Mouse</td>
<td>N/A</td>
<td>N/A</td>
<td>1:15,000</td>
</tr>
</tbody>
</table>

Table 2.4: Primary antigen details including supplier details and probing conditions.

Antigen validations experiments were conducted by way of negative and positive controls. Negative controls omitted the primary antibody to look for unspecific binding and noise, where positive controls were conducted using the recommended cell/protein on each data sheet, known to contain the desired protein. Conditions for the antigen detection stayed the same as those outlined in the above table.
CHAPTER 3: DISRUPTION OF KEY TRANSPORT AND REGULATORY PROTEINS IN RAT PLACENTA WHEN SUBJECTED TO MATERNAL INTERMITTENT FASTING
3.1 INTERMITTENT FASTING ANIMAL STUDY

3.1.1 DIETARY REGIMES, FOOD INTAKE AND MATERNAL WEIGHT GAIN DURING PREGNANCY

During gestation maternal food intake in IF rats and control counterparts was monitored over 24 hour periods from copulation to term (figure 3.1.1A). Daily food intake is quantified as grams of food consumed per 100g of bodyweight. Food consumption in IF rats was lower at every time point when compared to control (2 Way ANOVA, \( p<0.001 \)) which reflects the diet regimes of constant or intermittent food availability. For both diet regimes, a significant increase in food intake was recorded near term when compared to earlier gestation (2 Way ANOVA, \( p<0.01 \)).

Maternal weight gain in IF and control pregnant rats was recorded daily from copulation to term (figure 3.1.1B). Maternal weight gain (g) increased gradually as gestation progress in both diet regimes (2 Way ANOVA, \( p<0.001 \)) which reflects expected maternal weight gain due to fetal and placental growth. Despite weighing the same at copulation (E0) dams subjected to the IF diet regime gained weight at a slower rate throughout their pregnancy resulting in significantly lower maternal weight at term (2 Way ANOVA, \( p=0.02 \)).

3.1.2 EFFECT OF DIETARY REGIME ON FETAL AND PLACENTAL WEIGHTS

At E21, fetal and placental weights were recorded from litters of mothers that underwent IF or control diets. Mothers that underwent the IF regime had litters with increased fetal weights (figure 3.1.2A) and decreased placental weights (figure 3.1.2B; Mann-Whitney U, \( p<0.01 \)) compared to those from mothers on a control diet. Due to this, the fetal:placental weight ratio of pups from mothers under the IF regime was increased (figure 3.1.2C, Mann-Whitney U, \( p<0.01 \)) compared to mothers on control diets implying an increased fetal weight generated per gram of placenta.
Figure 3.1.1: (A) Daily maternal food intake in IF rats (black triangles, n=9) was significantly lower when compared to intake in control rats (grey squares, n=11) throughout gestation (E0-E21) (Mann-Whitney U, \( p<0.0001 \)). Food intake increased significantly in late gestation for both IF and control groups (2 Way ANOVA, \( p<0.01 \)). (B) Daily maternal weight gain in IF rats and control rats showed significant increases throughout gestation (E0-E21) (2 Way ANOVA, \( p<0.001 \)). Maternal weight gain in the IF diet regime was significantly lower when compared to control dams throughout gestation (2 Way ANOVA, \( p<0.05 \)). +/- SEM
Figure 3.1.2: The IF dietary regime (n=66) resulted in A) increased fetal weight ($p<0.01$), B) decreased placental weight ($p<0.01$) and C) increased fetal:placental weight ratio ($p<0.01$) at E21 compared to control counterparts (n=85) Statistical tests performed were Mann-Whitney U. +SEM.
3.1.3 PROTEIN CONCENTRATION OF RAT PLACENTAS

The total protein concentration from the cytoplastic and membrane fractions of rat placentas, from both IF and control diet regimes, was recorded at term (figure 3.1.3). The total protein concentration of the IF cytoplastic fraction was significantly lower than the cytoplastic fraction from control counterparts (Mann-Whitney U, \( p<0.05 \)). For both diet regime groups, the protein concentration of the membrane fraction is greater than corresponding cytoplastic fractions (Mann-Whitney U, \( p<0.05 \)).

3.1.4 PLACENTAL CONTENT OF TARGET PROTEIN: TRPV6, PMCA, SNAT2, PLACENTAL IGF-II AND CLOCK

The presence of placental proteins was quantified in the placental membrane fraction of IF and control samples (\( n=6/ \)group; figure 3.1.4). Protein is expressed as density (%) of Beta-Actin - a house keeping protein.

The presence of TRPV6, PMCA and SNAT2 is significantly decreased in the membrane fraction of IF samples when compared to samples which were subjected to the control diet regime (figure 3.1.4A, B, and D; Mann Whitney U, \( p<0.01 \)). Diet regime had no detectable differences on placental content of calbindin-D\(_{9k}\)proteins (figure 3.1.4C).

The presence of placental CLOCK protein is significantly increased in IF samples when compared to control counterparts (figure 3.1.5A; Mann Whitney U, \( p<0.05 \)). Diet regime had no detectable differences on placental content of calbindin-D\(_{9k}\) or IGF-II proteins (figure 3.1.5B).
Figure 3.1.3: Total protein concentration of cytoplasmic and membrane fractions from IF and control samples (n=6/group). For both diet groups, placental protein from cytoplasmic fractions was less than in membrane fractions (Mann Whitney U, p<0.05) Total protein concentration of the cytoplasmic fraction from IF samples was significantly lower than controls (Mann-Whitney U, p<0.05), however no effect of diet regime was seen between membrane fractions and maternal diet. The median is represented by the horizontal line.
Figure 3.1.4: Western blots quantified protein content of membrane fractions of placentas from litters of rats fed on control or IF dietary regimes. Representative Western blots are presented and all data were normalized against the house keeping protein β-Actin. IF reduces presence of placental TRPV6 (A), PMCA (B) and SNAT2 (D) in the rat when compared to control diet counterparts (Mann Whitney U, p<0.01; n=6). Maternal diet had no apparent effect on content of Calbindin-D9k in the cytoplasmic fraction (C; n=6). The median is represented by the horizontal line.
Figure 3.1.4: Western blots quantified protein content of membrane fractions of placentas from litters of rats fed on control or IF dietary regimes. Representative Western blots are presented and all data were normalised against the house keeping protein β-Actin. Placental content of CLOCK protein (A) was increased in placentas of IF fed rats compared to control diet (Mann-Whitney U, \( p<0.05; \ n=5 \)). Maternal diet had no apparent effect on content of IGF-II (B; \( n=6 \)). The median is represented by the horizontal line.
CHAPTER 4: THE IDENTIFICATION AND MANIPULATION OF CORE CIRCADIAN PROTEINS IN THE TROPHOBLASTIC CELL LINE: BEWO.
4.1 BeWo Viability Assay

Nuclei isolated from BeWo were visualised under a light microscope after staining with trypan blue (figure 4.1). Dark spots indicate isolated nuclei whereas cytoplasmic debris is identified as smears. No cytoplasmic debris was seen in the nuclei isolations indicating a high level of nuclei purity.

4.2 Quantification of CLOCK and BMAL1 in Nuclear and Cytoplasmic Fractions of BeWo

Nucleic and cytoplasmic BeWo fraction Western blot analysis to confirm the presence and localizations of key circadian regulators CLOCK and BMAL1 in the choriocarcinoma cell line BeWo is shown in figure 4.2.1. Both fractions displayed clear signals, however BMAL-1 does appear to be made up of several close bands, indicating the presence of CLOCK and BMAL1 in both the nuclei and cytoplasm of the choriocarcinoma cell line. Sample fractions were loaded alternately to allow for easy comparison and identification of immune signals.

Total protein content of the nuclei and whole cell lysate fractions isolated from BeWo cells cultured at 10, 20, 30 and 45mmol/l (n=3). Protein content was quantified as µg/µl. Figure 4.2.2 shows the change of glucose concentration in the growth medium does not affect the total protein concentration in either nuclear or whole cell fractions.
Figure 4.1: Visualisation of trypan blue nuclei quality assays (2.3.11) under a light microscope. BeWo were cultured in 10 (A), 20 (B), 30 (C), and 45 (D) mmol/l glucose concentration. Dark spots are representative of isolated nuclei whereas cytoplasmic debris are characteristic by smears, of which there are none.
Figure 4.2.1: Representative Western blots of the key circadian regulators (A) BMAL1 68kDa and (B) CLOCK 95kDa in both the nucleic and cytoplasmic fractions of the choriocarcinoma cell line BeWo. Clear signals indicate that the circadian machinery is localised in both fractions.
Figure 4.2.2: Protein concentration of both nucleic (A) and cytoplasmic (B) fractions isolated from BeWo cultured at varying glucose concentrations; 10, 20, 30 and 45mmol/l. Protein is displayed as µg/µl (n=3). The median is represented by the horizontal line.
The presence of circadian machinery proteins was quantified and compared in nuclear and cytoplasmic fractions were cultured in 10, 20, 30 and 45 mmol/l glucose concentrations in BeWo (n=3; figure 4.2.3). Western blot analysis of circadian machinery was normalised against the house keeping protein Beta-Actin and displayed as a density (%). Densitometric analysis shows changed protein content of trophoblastic CLOCK and BMAL1 with increasing concentrations of glucose in the media. CLOCK significantly decreases between 10 and both 30 and 45mmol/l glucose concentrations in nucleic fractions (A), and between 10 and 45mmol/l in cytoplasmic fractions (Kruskal-Wallis with Dunn’s multiple comparisons, p<0.0001). Nuclear (C) and Cytoplasmic (D) content of BMAL1 protein is significantly increased at 10mmol/l compared to all other glucose concentrations (One-way ANOVA Tukeys post hoc, p<0.01). No differences were observed between 20-45 mmol/l media glucose concentrations. β-Actin expression remained unchanged throughout glucose concentration changes.

CLOCK and BMAL1, quantified in nucleic and cytoplasmic fractions of BeWo cultured in 10 (A), 20 (B), 30 (C) and 45 (D) mmol/l glucose media concentrations was shown in figure 4.2.4. Densitometric analysis shows no significant difference between cytoplasmic and nucleic content of CLOCK or BMAL at each glucose concentration (Mann Whitney U).
Figure 4.2.3: The content of circadian machinery proteins, CLOCK and BMAL1, were quantified in the nucleic and cytoplasmic fraction of BeWo cells cultured in 10, 20, 30 and 45mmol/l glucose (n=3). All data were normalised against the house keeping protein β-Actin. CLOCK significantly decreases between 10 and both 30 and 45mmol /l glucose concentrations in nucleic fractions (A), and between 10 and 45mmol/l in cytoplasmic fractions (Kruskal-Wallis with Dunn’s multiple comparisons, p<0.0001). BMAL1 is significantly higher in 10mmol /l compared to 20 mmol /l glucose concentrations in both the nucleic (C) and cytoplasmic (D) fractions (Kruskal-Wallis with Dunn’s multiple comparisons, p<0.001 and 0.05 respectively). +/- SEM.
Figure 4.2.4: The expression of CLOCK and BMAL1 in nuclear (N) and cytoplasmic (Cy to) fractions of BeWo in (A) 10, (B) 20, (C) 30 and (D) 45mmol/l glucose concentration in the culture medium. There were no differences between nuclear and cytoplasmic CLOCK or BMAL1 expression in any glucose concentration. +/- SEM.
4.3 Stimulation of Cyclic CLOCK and BMAL1 Feedback Loops in BeWo

4.3.1 Cell Viability Following Serum Shocking

Cell viability MTT assays were performed on BeWo before (0, n=12), directly after the 2 hour serum shock (2, n=31) and 48 hours (48, n=36) after the addition of serum free media (figure 4.3.1). MTT metabolism during the cell serum shocking procedure resulted in no significant difference in the reduction of MTT to the insoluble form when compared to pre serum shocked counterparts.

4.3.2 Measuring of Cyclic Activity of CLOCK and BMAL1

The expression of the key circadian regulators CLOCK and BMAL1 were monitored in BeWo every 6 hours for a period of 48 hours after the serum shock procedure and in control samples using Western blotting (figure 4.3.2). Results are displayed as a density (% of β-Actin). Expression of CLOCK and BMAL1 in control tissue (A) recorded significantly higher levels of CLOCK compared to BMAL1 counterparts. After the serum shock procedure (B) the expression of CLOCK is significantly reduced compared to control samples (one way ANOVA, p<0.05) and the expression levels recorded closely match BMAL1 expression in serum shocked samples.

4.3.3 Levels of Calcium Transport Proteins, TRPV6 and PMCA, in Serum Shocked BeWo

The expression of the key calcium transporters TRPV6 and PMCA was monitored in BeWo every 6 hours for a period of 48 hours after the serum shock procedure and in control samples using Western blotting (figure 4.3.3). Results are displayed as a density (% of β-Actin). Expression of TRPV6 in control and serum shocked samples (A) displayed no significant difference at any time point. The expression of PMCA in serum shocked samples and control counterparts (B) similarly showed no variation after the serum shock procedure.
Figure 4.3.1: Cell viability as measured by the MTT assay shows no difference between the reduction of MTT at time 0 (n=12) or 48 (n= 36) hours post serum shock procedure. +/- SEM.
Figure 4.3.2: The expression of CLOCK and BMAL1 in BeWo measured via Western blotting in control samples (A) and after the serum shock procedure (B) (n=3/time point). Expression was recorded every 6 hours for a period of 48 hours post serum shock. CLOCK expression was significantly reduced after serum shocking when compared to control counterparts (One way ANOVA, p<0.05). +/- SEM.
Figure 4.3.3: The expression of TRPV6 (A) and PMCA (B) in BeWo measured via Western blotting in control samples and after the serum shock procedure (n=3/time point). Expression was recorded every 6 hours for a period of 48 hours post serum shock. TRPV6 and PMCA expression is unaltered after the serum shock procedure. +/- SEM.
4.3.4 **Levels of System A Transport Protein, SNAT2, in Serum Shocked BeWo**

The expression of SNAT2 was monitored in BeWo every 6 hours for a period of 48 hours after the serum shock procedure and in control samples (n=3/time point; figure 4.3.4). Results are displayed as a density (% of β-Actin). The levels of SNAT2 protein in control displayed no significant difference post serum shocking.
Figure 4.3.4: The expression of SNAT2 in BeWo measured via Western blotting in control samples and after the serum shock procedure (n=3/time point). Expression was recorded every 6 hours for a period of 48 hours post serum shock. SNAT2 expression displays no significant difference after the serum shock procedure (n=3/time point). +/- SEM.
CHAPTER 5: DISCUSSION
The rat model of IF presented in this study shares characteristics seen in human pregnancy during Ramadan with regards to pregnancy outcome. The rat placenta was reduced in size following maternal IF in common with observations in humans during a period of Ramadan type fasting (Alwasel et al. 2010). The IF during the gestational period in the rat also brought about significant changes in the protein expression of key calcium and amino acid transporters in the placenta. This period of IF also altered the protein expression of the key circadian regulatory protein CLOCK in the rat placenta. It is not clear whether this was due to the abnormal dietary cues or the disrupted sleeping pattern which came about as a result of IF in the rat. The circadian machinery was shown to be expressed in the human choriocarcinoma cell line BeWo which is a model of the syncytiotrophoblast. However, a serum shock procedure was unable to stimulate circadian oscillations in the circadian regulators CLOCK and BMAL1 as it has previously been reported to do in another placental model clock genes (Frigato et al. 2009). However it did act to bring the protein expression levels of CLOCK and BMAL1 into close association. The altered expression of CLOCK combined with the serum shock procedure did not affect the expression of key nutrient transporting proteins for calcium and amino acids in BeWo.

The in vivo IF model investigated in this report was able to mimic certain outcomes reported in human pregnancy. The decrease in placental size in the rat during IF mirrors a similar decrease seen in Saudi Arabia during human gestation (Alwasel et al. 2010). It was reported that placental size was decreased in the second or third trimester of human pregnancies \( (p<0.002 \text{ for both boys and girls}) \) when subjected to Ramadan fasting compared to non-fasting counterparts. Despite the reduction in size, the placenta was able to maintain normal fetal development and to this end birth weight was unaffected (Alwasel et al. 2010). The IF rat model produced an increased fetal birth weight as well as decreased placental size at term. Previous papers have alluded to a placental reserve capacity that is able to increase the efficiency of the placenta per gram of placental tissue when environmental stressors occur during pregnancy (Baergen 2007; Coan et al. 2008; Fox 1997). This reserve capacity has previously been investigated with regards to placental morphology. The authors found
that volume of labyrinthine zone in mouse placenta was increased at E16 in the smallest placenta from each sampled litter coupled with up-regulated expression of the SNAT2 gene E19 (Coan et al. 2008). It may be that the IF period during gestation causes a big enough insult to fetal development that the placenta calls upon the reserve capacity to maintain fetal growth at a normal trajectory in the rat model. The increased fetal weight recorded in the IF rat model could be a result of the larger fasting period during the whole gestational period. If fasting were to happen in human gestation for the same period it may well be that similar results occur.

Following gestational IF, the expression of key calcium transporting proteins and SNAT2 in the rat placenta was significantly lower than controls. The decrease in the protein expression of TRPV6, PMCA and SNAT2 is counter to the afore reported labyrinthine zone volume expansion in smaller placentas (although no morphological studies were conducted for this particular IF study) and the SNAT2 amino acid transporting gene up regulation in the mouse (Coan et al. 2008). The decrease in TRPV6 protein expression at term in the rat placenta following IF could affect calcium flux from maternal to fetal compartments. Work conducted using the TRPV6 knockout mouse recorded a significant decrease in calcium transport across the placenta (Suzuki et al. 2008). It is possible that the IF rat model could be associated with a decrease in calcium transport from the maternal circulation due to the decrease in TRPV6 protein expression. It has also previously been reported that, in the rat, the activity of PMCA does not mirror its expression (Nabekura et al. 2004). It may be that this is the case with the down regulated nutrient transporter protein expression observed in the current study and, an increase in activity may make up for the decrease in protein expression. In a previous study looking at pre-eclampsia and the affect it had on placental nutrient transporters in human gestation the authors reported an increase in TRPV6 and PMCA expression on both the maternal and fetal side of the placenta (Yang et al. 2013). This is at odds with the changes seen in our IF study, however it should be taken into consideration the difference between a controlled period of fasting and a pre-eclampsia pathology. Interestingly, the protein levels of Calbindin-D_{9k} remain unchanged in the rat
placenta at term after a period of IF. In the past it has been suggested that Calbindin-D\textsubscript{9k} is rate limiting for calcium transport across the placenta in rodents (Dilworth et al. 2010; Bond et al. 2008; Glazier et al. 1992). It could be that the effect of reduced protein levels of TRPV6 and PMCA is minimal due to sufficient calcium shuttling through the trophoblastic layers via unaltered levels of Calbindin-D\textsubscript{9k} in the rat model.

The amino acid transporting protein SNAT2 was identified before the study as a good indicator of dietary alteration to nutrient transporters due to its well documented ability to be extremely adaptable under nutritional challenge (Dall'Asta et al. 1994a; Franchi-Gazzola et al. 1999; Franchi-Gazzola et al. 1994; Jones et al. 2006a; Novak et al. 2006). Indeed a period of IF significantly reduced the expression of the SNAT2 protein in the rat placenta. This indicates that IF during gestation caused a large enough nutritional challenge for the placenta to respond. The reduction in SNAT2 protein expression in the placenta is counter logical to the increase in fetal body weight recorded at term. Similar to calcium transporters, it could be that the activity of the SNAT2 transporter protein could be increased to cope with the decrease in protein expression. Taken together, the decreased protein expression of placental TRPV6, PMCA and SNAT2 indicate a change in nutrient transport activity that eventually leads to the increased fetal body weight at term in our rat IF model.

The increase in placental CLOCK after a period of IF in the rat suggested that the alteration to normal animal housing conditions over the gestational period (feeding during light hours) changed the circadian function in the placenta. It also acted to challenge the idea that the placenta has a more robust mechanism to contend with the cyclical nature of peripheral tissues (Waddell et al. 2012). The change in maternal feeding patterns is clear in the IF rat model utilised in this study and it could be that the placenta is just as susceptible to these dietary cues as other peripheral tissues. It could be postulated that it is the circadian disturbance rather than the nutritional mismatch that causes the effects on pregnancy outcome. Recent studies have shown that a simple light manipulation of maternal housing conditions can reduce fetal growth in the rat by up to 24% (Gozeri et al. 2008). The nature of the IF fasting period used in this study affects the nocturnal nature of the rat as they were
denied food from 5pm until 9am. The IF rat reported in this model had to contend with altered dietary cues as well as altered light exposure due to the disruption in their waking hours.

It must be taken into consideration that the IF rat model used here has a number of limitations. Firstly the whole gestational period; is subjected to the fasts whereas the Ramadan period would last no longer than 1 month of the human gestation. Secondly, water was freely available to rats during the IF period, during Ramadan water intake is also restricted in waking hours. Thirdly, protein expression may be slightly misleading when taken alone and future work should look at transporter protein activity. Lastly, although the IF model started to address the possibility that circadian cues play an important role during placental regulation in pregnancy it must be noted that the expression of CLOCK protein alone gives very little information on the physiology of circadian regulation. Therefore the relationship between the circadian clock in the placenta and nutrient transfer between mother and fetus remains unknown.

Localisation of circadian machinery to the syncytiotrophoblast in both the nuclear and cytoplasmic fractions of the choriocarcinoma cell line BeWo and the subsequent stimulation of CLOCK and BMAL1 expression in BeWo using asynchronous dietary changes of glucose concentration led to altered protein expression in both the nucleic and cytoplasmic fractions. Glucose and its ability to disrupt CLOCK and BMAL is well documented, however the complex nature of glucose homeostasis and insulin action means the mechanisms remain unknown (Marcheva et al. 2010). The ability of an asynchronous dietary cue to alter the expression of key circadian proteins in the trophoblast alludes to a possibility that any altered glucose homeostasis during pregnancy may affect circadian control in the placenta. Very few studies have been conducted to look at placental phenotype with regards to targeted disruption of the clock genes. One such model, the BMAL1 knockout mouse, was found to be smaller and up to 30% lighter at birth than control pups (Boden et al. 2010; Kondratov et al. 2003). The mechanisms behind BMAL1 knockout mouse growth restriction remain unknown; it could be that the deletion of BMAL1 perturbs the cyclic nature of the clock which
has been shown to govern nutrient homeostasis in other tissues (Marcheva et al. 2010; Wharfe et al. 2011).

In order to elucidate the relationship between cyclical circadian oscillations and nutrient transport the serum shock procedure was used. Serum shocking is widely used in cell culture applications to investigate circadian expression of various genes (Balsalobre et al. 1998; Matsunaga et al. 2014). In the circadian clock BMAL and CLOCK interact together to form a heterodimer in the cytoplasm and shuttle back into the nucleus to inhibit their own action in the TTO model. The serum shock procedure did not result in an oscillation of CLOCK and BMAL1 protein expression in the BeWo cell line. However the 48 hour period post serum shock showed a significant decrease in BeWo CLOCK expression. As the circadian elements undergo post transcriptional changes and interactions in the cell cytoplasm (Waddell et al. 2012), it could be that these modifications are fundamentally different in the placenta. This conclusion is supported by evidence that the BMAL1 and CLOCK cyclical phase shows no difference to other core clock genes Per1 and Per2 in the rat placenta which is known to be the anti-phase of the BMAL-CLOCK loop (Wharfe et al. 2011). Both core clock loops were shown to have parallel peaks during the dark phase which indicates an altered circadian mechanism in rodent placenta. The decrease in the CLOCK expression in the serum shocked BeWo samples did act to bring the CLOCK expression levels into close association with BMAL1 levels. Considering the close relationship between CLOCK and BMAL1 in the TTO feedback loop the similar expression levels come as no surprise. BMAL1 expression showed no significant difference after the serum shock procedure and this may be more telling as it is considered to be the more influential half of the CLOCK-BMAL1 complex due to the E-Box element. This decrease in CLOCK expression which happened as a result of the serum shock did not bring about significant changes in nutrient transporters, it has long been suggested that the placenta has a more robust mechanism to cope with circadian oscillations due to the nature of the organ and this maybe evidence to support that theory.
The IF rat model was able to mimic observations in women partaking in Ramadan with regards to placental size. The mechanisms behind placental function were investigated and it was shown that IF during gestation reduced the expression of key calcium transporting proteins and the amino acid transporting protein SNAT2. The period of fasting also brought about with increased expression of the key circadian regulatory protein CLOCK. Further investigation into circadian control of placental nutrient transport provided insight into the localisation of circadian machinery to the key nutrient transporting cells of the human placenta. The serum shock procedure was unable to stimulate circadian oscillations in the placental cell line BeWo; however it did act to reduce the expression of CLOCK. This reduction in CLOCK did not act to alter the expression of TRPV6, PMCA or SNAT2 in BeWo but it could be that the isolated cell line lacks the necessary components for total control of the circadian clock. Further work needs to be conducted to elucidate to what extend disruptions to the circadian clock can control nutrient transport and homeostasis, possibly with further use of the rat model. The ability of the asynchronous dietary cues to influence circadian protein expression indicates a complex relationship between circadian control and nutrient homeostasis. Although this report was unable to identify a direct link between the two, further investigations may look at a complete organism rather than isolated cell line and to what extend the circadian clock influences and controls the function of key nutrient transporting tissues. Data reported above is an important first step as it provides information into the fundamental changes that happen in key nutrient transporting proteins during a period of fasting in the placenta. It also tentatively begins to explore a link between circadian control and nutrient transport.

6.2 Future Work

The decrease seen in the protein expression of TRPV6, PMCA and SNAT2 suggests the activity must be increased to compensate and maintain fetal growth. From current data this can only be speculated, therefore experiments to measure transporter activity must be conducted. The maternal and placental clearance of radio labelled transporter substrates
such as Ca$^{2+}$ and amino acids could be used to assess transporter protein activity during IF. This would provide further information on the placentas ability to cope with nutritional insult in utero (Constância et al. 2005).

Isolation of specific placental zones from the rat model could provide more information with regards to circadian protein expression and the relationship with nutrient transporter protein expression throughout pregnancy. This study looked at whole expression of placental circadian machinery in the rat. Zone specific changes may offer further insight into circadian and nutrient homeostasis.
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