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Regulation of Glucose Transporters in Sheep Placenta

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Auckland, Auckland, New Zealand

February 2001
ABSTRACT

Transplacental glucose transport is vital to fetal growth. Although the presence of glucose transporter-1 (GLUT1) and GLUT3 has been demonstrated in mammalian placenta, the factors regulating these genes remain unclear. Therefore, the overall aim of these studies was to clone ovine GLUT1 (oGLUT1) and oGLUT3 cDNAs, and to use these to investigate gene expression during ovine placental development and function.

Ovine GLUT1 (~2.2 kb) and oGLUT3 (483 bp) cDNAs were isolated and cloned. Sequence analysis demonstrated that oGLUT1 showed high homology (97 – 99%) with other mammalian species, whereas oGLUT3 did not (84 – 88%).

Northern analysis demonstrated that oGLUT1 mRNA abundance increased from d 45 to d 120 of gestation, then decreased towards term (d 145 ± 2), whereas oGLUT3 mRNA abundance increased throughout gestation. Western analysis showed oGLUT1 protein levels increased during late gestation, indicating post-transcriptional regulation of oGLUT1.

Localisation experiments revealed spatio-temporal differences in ovine placental GLUT expression. In early gestation (d 45), oGLUT1 protein was restricted to fetal trophoblast cells. By mid gestation oGLUT1 immuno-signal was predominantly localised to maternal villous and endometrial tissue. By late gestation oGLUT1 mRNA was most strongly localised to maternal syncytiotrophoblast and villous tissue, whereas oGLUT3 was predominantly localised to fetal trophoblast cells.

Placental oGLUT expression was regulated differently by acute (3 – 8 h) versus long-term (> 6 d) alterations in late gestation maternal glucose supply. No evidence was found for regulation of placental oGLUT gene expression by long-term maternal undernutrition, but oGLUT1 and oGLUT3 mRNA and oGLUT1 protein were elevated by short-term (24 – 48 h) maternal hypoglycemia. Acute maternal hyperglycemia transiently increased oGLUT1 and oGLUT3 mRNA abundance, whereas oGLUT1 protein (but not mRNA) levels increased after long-term maternal hyperglycemia.

Infusion studies provided no conclusive evidence for regulation of placental oGLUTs by long-term administration of growth hormone (GH) or insulin-like growth factor-1 (IGF-1) to the late gestation fetus. Following acute (4 h) fetal IGF-1 infusion, placental oGLUT3 mRNA
abundance was greater in growth restricted (placental embolisation) than in normal fetuses, although the reason for this difference remained equivocal.

This thesis describes isolation, cloning and sequence analysis of oGLUT1 and oGLUT3 cDNAs. These studies confirmed the presence of GLUT1 and GLUT3 mRNA in ovine placenta, and demonstrated ontogenetic and nutritional regulation of placental oGLUT1 and oGLUT3. In addition, these results indicated that regulation of placental oGLUTs may occur at both transcriptional and post-transcriptional levels.
ACKNOWLEDGEMENTS

I would like to thank everyone who has helped during the course of my PhD.

My supervisors, Dr Nicole Bassett, without whom this thesis would never have been started, and Professor Jane Harding, whose advice and encouragement in the final months were invaluable.

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To all my friends and family, who have supported me throughout this study.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Ad Lib</td>
<td>ad libitum</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon nuclear translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BM</td>
<td>basal membrane</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>BNC</td>
<td>binucleate cells</td>
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<td>cyclic adenosine-3',5'-monophosphate</td>
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<td>carbonate buffered saline</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>cpm</td>
<td>counts per minute</td>
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<tr>
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<td>cytidine-5'-triphosphate</td>
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<tr>
<td>d</td>
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<td>ddH₂O</td>
<td>double distilled water</td>
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<td>dalton</td>
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<td>3'5' dianaminobenzidine</td>
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<td>diethyl pyrocarbonate</td>
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</tr>
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<td>salmon sperm DNA</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide mix</td>
</tr>
<tr>
<td>DPX</td>
<td>distrene, dibutyl phthalate, xylene (mounting media)</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FFA</td>
<td>free fatty acid</td>
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<tr>
<td>FRM</td>
<td>rich bacterial medium</td>
</tr>
<tr>
<td>g</td>
<td>gram (weight) or gravity (centrifugation)</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>GLUT</td>
<td>glucose transporter</td>
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<tr>
<td>GTE</td>
<td>glucose, Tris, EDTA</td>
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<td>guanidine-5'-triphosphate</td>
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<td>HEPES</td>
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<tr>
<td>IGF</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IM</td>
<td>internal membrane protein fraction</td>
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<td>ISH</td>
<td>in situ hybridisation</td>
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<td>IPTG</td>
<td>isopropyl-β-D-thio-galactopyranoside</td>
</tr>
<tr>
<td>IUGR</td>
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</tr>
<tr>
<td>k</td>
<td>(prefix) kilo- (10³)</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>µ</td>
<td>(prefix) micro- (10⁻⁶)</td>
</tr>
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<td>µCi</td>
<td>microCurie</td>
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<tr>
<td>m</td>
<td>(prefix) milli- (10⁻³)</td>
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<td>MOPS</td>
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<td>MVM</td>
<td>microvillous membrane</td>
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<td>molecular weight</td>
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<td>NIDDM</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>plaque forming units</td>
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<td>PM</td>
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<td>Research Centre for Developmental Medicine and Biology</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>standard error of the mean</td>
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<td>saline sodium phosphate EDTA buffer</td>
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<td>streptozotocin</td>
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<td>TBS</td>
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<td>tris EDTA buffer</td>
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<tr>
<td>TTP</td>
<td>thymidine-5'-triphosphate</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UN</td>
<td>undernutrition</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine-5'-triphosphate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>w/w</td>
<td>weight to weight ratio</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 GENERAL INTRODUCTION

Fetal growth and development determine survival and health
In humans, abnormal fetal development may cause anatomical, metabolic and neurological changes that are associated with increased risk of perinatal mortality and morbidity. Postnatally, such individuals are prone to cardiovascular, metabolic and skeletal defects (Gillmer et al., 1984), cerebral palsy (Blair and Stanley, 1990), intellectual delay, and behavioural problems (Rantakallio, 1985; Taylor and Howie, 1989). Furthermore, recent epidemiological studies have defined close associations between abnormal growth of the fetus and infant with the risk of developing adult-onset diseases such as coronary heart disease, diabetes mellitus (type II), hypertension and chronic lung disease (Barker, 1990). Thus, fetal growth and development have widespread and life-long ramifications for the health of the individual.

Nutrient supply is the primary determinant of fetal growth
The rate and pattern of fetal growth and development are determined by interactions between the inherited genetic potential for growth, and the availability and utilisation of substrates essential for growth (Robinson and Owens, 1996). In the constrained environment of the uterus the fetus is dependent upon a continuous supply of nutrient substrates provided by the mother to the conceptus (uteroplacenta and fetus), and by the uteroplacenta (a group of tissues including the myometrium, endometrium, placental trophoblast mass, and the extraplacental chorionic membrane) to the fetus (Battaglia and Meschia, 1986). Therefore, under normal conditions, a major determinant of fetal growth and development is placental nutrient transfer to the fetus.

Maternal glucose is a vital nutrient for fetal growth
Glucose is an important fetal nutrient. It is a major source of carbon for the growing fetus, providing substrate for both tissue accretion (growth) and cellular energy metabolism (survival) (Battaglia and Meschia, 1988). Most tissues use glucose preferentially, and some (eg, brain) are obligate glucose users (Jones and Rolph, 1981). In adults, glucose may be acquired through nutrient intake, released from glucose storage molecules such as glycogen (glucogenesis), or produced de novo from other carbon containing molecules (eg, amino acids) by the process of gluconeogenesis. However, the fetus has few energy reserves, and is unable to produce its own glucose supply until late gestation when key gluconeogenic enzyme pathways mature and allow limited glucose production (Jones and Rolph, 1985). Therefore,
maternal glucose supply transported to the fetus via the placenta is practically the sole source of glucose available to the fetus, and is vital to normal growth and development.

Placental glucose transport occurs via plasma membrane spanning transporter proteins
Transplacental glucose transport occurs by facilitated diffusion (Johnson and Smith, 1980). As with simple diffusion, the direction of glucose transport is determined by movement of glucose down its concentration gradient. However, the rate of facilitated diffusion is increased many thousandfold by membrane-spanning glucose transporter (GLUT) proteins that facilitate the movement of glucose across the placental membranes (Mathews and van Holde, 1990). Thus, regulation of placental glucose transport may be affected by factors that alter:

- Glucose concentration gradients across the maternal- and fetal-facing placental plasma membranes (eg, maternal glucose availability, placental glucose metabolism, and fetal glucose utilisation)
- Presence and/or activity of GLUT proteins in placental membranes.

Molecular physiology of placental glucose transport is unclear
Experimental models of pregnancy using chronic catheterisation and in vivo tracer methodology have elucidated many of the maternal, placental and fetal factors that alter transplacental glucose concentration gradients and direct the flux of glucose to the fetus during pregnancy (Harding and Johnston, 1995; Hay, 1995a; Owens, 1991). However, the molecular regulation of placental GLUT proteins, and the extent to which their regulation may influence fetal glucose supply is not yet clear. Ultimately, a thorough understanding of placental nutrient transport may allow development of molecular therapies to optimise fetal nutrient supply in cases of poor fetal growth.

General thesis aims and overview
The overall aim of this thesis was to investigate molecular regulation of placental glucose transport. The pregnant ewe has been widely used for the study of nutrient transfer during pregnancy. Therefore, this model was selected so that the current studies on placental GLUT molecular regulation might be compared with complementary in vivo studies investigating maternal-fetal nutrient transfer and placental function. Because ovine probes were not available when these studies began, the first priority was to generate ovine specific cDNAs for the two placental GLUT isoforms known to be predominant in human and rodent placenta, namely GLUT1 and GLUT3 (Gould and Holman, 1993) (Chapter 3). These cDNAs were then used to characterise normal GLUT mRNA abundance and location in ovine placenta at
selected gestational ages, and to establish the presence and location of ovine GLUT1 (oGLUT1) and ovine GLUT3 (oGLUT3) mRNA in ovine placenta from early (d 45) to late (d 138) gestation (term d 145 ± 2, Chapter 4). The final studies investigated regulation of placental oGLUT1 and oGLUT3 gene expression by factors known to alter glucose flux to the fetus in late gestation, namely maternal undernutrition and glycemia (Chapter 5), and fetal insulin-like growth factor-1 (IGF-1) (Chapter 6).

The remainder of this introductory chapter reviews the literature relevant to placental glucose transport. Major themes include development of placental nutrient transfer function; maternal, placental and fetal factors influencing transplacental glucose transfer; and developmental regulation of placental GLUT1 and GLUT3 abundance and localisation. Chapter 1 concludes with a review of thesis aims, hypotheses and chapter topics. Chapter 2 describes general methods and materials used in these studies. Chapters 3 – 6 present the experimental studies including specific methods, results, and discussion. Chapter 7 discusses the overall significance of these findings and potential future directions for this research.
I. FETAL GROWTH AND UTEROPLACENTAL GLUCOSE TRANSFER

Growth is regulated by interactions between the genome, and nutritional and endocrine factors that determine the availability and utilisation of nutrient substrates (Gluckman and Heyman, 1996). Generally, the individual’s inherited genetic potential is the primary determinant of growth. However, in the constrained environment of the uterus, fetal nutrient supply is limited to substrates supplied from the mother via the uteroplacenta (Beard and Nathanielsz, 1984). Uteroplacental nutrient transfer is therefore the major determinant of fetal growth.

The placenta is the organ of exchange via which uteroplacental nutrient transfer to the fetus occurs (Battaglia, 1986; Johnson and Smith, 1980). Although placental morphology differs among mammalian species, a common structure is the syncytiotrophoblast. The syncytiotrophoblast is a multinucleate layer of fused epithelial cells that forms the functional barrier to transfer of nutrients between mother and fetus (Sibley et al., 1998). The sites of most known placental cellular transport mechanisms are localised to the plasma membranes of the syncytiotrophoblast. Flux across the maternal- and fetal-facing syncytiotrophoblast plasma membranes represents the rate-limiting step in maternal-fetal transfer of most important fetal nutrients (Smith et al., 1992).

During gestation, three stages of placental development occur: implantation, growth and maturation (Hay and Wilkening, 1994). The first two stages occur early in gestation, and placental growth, as defined by increase in size and/or weight, is largely achieved in the first half of gestation. In contrast, fetal growth is greatest, and therefore most demanding of placental nutrient supply, in late gestation (Hay and Wilkening, 1994; Schneider, 1996). These markedly different temporal patterns of growth have important implications for the ability of the placenta to supply sufficient nutrients to the late gestation fetus, and necessitate the third phase of placental development – maturation of placental nutrient transfer function.

This section will describe placental morphology, and review placental growth and development in the context of placental nutrient transfer function.
1.2.1 Placental morphology

The majority of studies on placental glucose transport described in this review have been carried out in humans, rats and sheep. Therefore, this section describes placental morphology in all three species.

1.2.1.1 Comparative placental morphology

The placenta and fetus both arise from the single cell of the fertilised ovum. Hence, the placenta is of fetal origin. Placental implantation is initiated when the outer trophoblast cells (chorionic epithelium) of the blastula attach to the epithelium of the maternal uterine mucosa, and interact with or invade this tissue to varying (and species specific) degrees (Hay, 1996).

A number of structural characteristics have been used to classify placental morphology including gross morphology (diffuse, cotyledonary, zonary and discoid), surface area (compact or diffuse), and perfusion pattern (concurrent or countercurrent) (Dawes, 1968; Hay, 1996; Steven, 1975). With the advent of light microscopy, Grosser (1909) proposed a histological classification based on the number of layers of tissue separating fetal from maternal circulation. Six layers were included: maternal blood vessel endothelium, maternal connective tissue, and maternal epithelium; and fetal epithelium, fetal connective tissue and fetal blood vessel endothelium. Recent techniques have demonstrated that in all species studied there is a wide range of structural variation among placentas, and within each placenta different layers may be present in various regions or at various times during gestation (Steven, 1975). However, a modified form of Grosser's classification is still widely used, with three major categories of interaction between the placenta and the uterine mucosal epithelium now recognised (Figure 1.1) (Dawes, 1968; Hay and Wilkening, 1994; Steven, 1975). (Grosser published in German – the original reference is included in the review by Dawes (1968)).
Chapter 1

Figure 1.1 Diagram of the three main anatomical types of placenta, classified according to histological structure. A Epitheliochorial (sheep, pigs and cattle), fetal and maternal endothelium and epithelium present. Maternal epithelium apposes chorionic epithelium or cytotrophoblast. B Endotheliochorial (Carnivora), fetal and maternal endothelia appose a syncytiotrophoblast. C Hemochorial (Rodenta, most Primates), the trophoblast erodes the maternal epithelium and endothelium, allowing maternal blood to directly bathe the maternal-facing microvillous membrane of the trophoblast. This category has been divided into the following three subcategories: C1 Hemomonochorial (human, guinea pig), human placenta has a chorion that consists of an outer syncytial and an inner cytotrophoblast layer. However, it is placed in the hemomonochorial category because the cytotrophoblast (or Langhan’s layer) is discontinuous and does not intervene between the fetal capillaries and the syncytial trophoblast, at least in late gestation. C2 Hemodichorial (rabbit). C3 Hemotrichorial (rat, mouse), in the rat, three layers of trophoblast separate the maternal blood spaces from the fetal blood vessels. The outer layer is cellular and fenestrated (cytotrophoblast), while the middle and inner are syncytial.

MB, maternal blood; FB, fetal blood; ME, maternal endothelium; BM, basement membrane; CT, connective tissue; UE, uterine epithelium; MV, maternal epithelial microvilli; TRV, trophoblast microvilli; CTR, cytotrophoblast; CE, chorionic epithelium; FE, fetal endothelium. Adapted from Hay (1996) and Hay and Wilkening (1994).
1.2.1.2 Ovine placental morphology

The gross appearance of mammalian placentae differs greatly in different species. However, all are composed of small fetal units containing chorionic villi, called cotyledons. In the human, implantation produces a single trophoblast tissue mass and cotyledons are combined to form a single flat disc. This shape accounts for the derivation of the word ‘placenta’ from the Greek word for ‘cake’ (Dawes, 1968). In sheep, diffuse implantation produces multiple small masses of trophoblast tissue, and the cotyledons (30 – 90 in number) are dispersed across the uterus. The points of materno-fetal attachment are provided by specialised areas of maternal tissue (endometrial folds) known as ‘caruncles’. The interdigitation of fetal cotyledon and maternal caruncle forms the exchange unit known as the ‘placentome’ (Steven et al., 1981). Cotyledon is derived from the Greek word for cup (kotyle) (Dawes, 1968), which describes the manner in which the cotyledon forms the concave inner surface of the ovine placentome (see Figure 1.2).

![Diagrammatic representation of an ovine placentome in the last trimester.](image)

**Figure 1.2** Diagrammatic representation of a vertical section through an ovine placentome in the last trimester. CD, central concavity of cotyledon; ChA, chorio-allantois; str, endometrial stroma (Steven et al., 1981).

Ovine placentome morphology varies according to the degree of eversion, or growth of the fetal cotyledon over the maternal caruncle. Placentomes may be classified as type A (inverted) to type D (everted) with types B and C being intermediate (Alexander, 1964; Heasman et al., 1998; Vatnick et al., 1991).

A unique feature of the ruminant placenta is the presence of fetal chorionic binucleate cells (BNC). These cells have two main functions: to form the feto-maternal syncytium, and to produce a number of peptide and steroid hormones (including ovine placental lactogen and progesterone) (Wooding et al., 1996). Binucleate cells appear to arise from division of the
nuclei but not the cytoplasm of the principal cells of the trophoblast (Steven et al., 1981). They constitute 15 – 20% of the chorionic epithelial cells throughout gestation until one or two days before parturition, when there is a rapid decrease. When mature, they migrate from the trophectoderm through the apical chorionic tight junctions to fuse with uterine epithelial cells, thus modifying the uterine epithelium into a hybrid feto-maternal syncytium (Wooding et al., 1992). Though single cells are occasionally evident, most of the uterine epithelium becomes a partial or incomplete syncytium, which takes the form of contiguous masses of multinucleate cytoplasm and is called the syncytiotrophoblast (Steven et al., 1981). The syncytiotrophoblast layer in hemochorial and endochorial placentas is continuous. In sheep, the syncytiotrophoblast takes the form of syncytial plaques, each limited to 20 – 25 nuclei and linked by tight junctions. Wooding et al. (1992) have proposed that a more accurate term for ruminant placenta is ‘synepitheliochorial’. The prefix ‘syn’ signifies fusion and emphasises the role of cell fusion in formation of the syncytiotrophoblast, yet this classification confirms the presence of both maternal and fetal epithelial layers, even though one (the uterine epithelium) is a feto-maternal hybrid.

No nuclear division or mitotic processes have ever been observed in ovine syncytiotrophoblast, so BNC migration is crucial to the enormous growth in area of this feto-maternal interface during gestation development (Wooding et al., 1992). In contrast to the syncytial changes that occur in the maternal epithelium, the fetal chorionic epithelial cells retain their cellular structure. They are cuboidal in shape with large nuclei (Steven et al., 1981).

1.2.2 Placental versus fetal growth

The pattern of placental and fetal weight increase during gestation shows general similarities in different mammalian species. The weight curves for placenta and fetus in sheep are illustrated in Figure 1.3 (Hay, 1996; Owens and Robinson, 1988; Schneider, 1996).

Placental growth is most rapid in early gestation. In ovine placenta, protein and DNA content are maximal by d 75 – 80 of gestation (Ehrhardt and Bell, 1995), and the placental cotyledons weigh approximately three times as much as the fetus (Battaglia and Meschia, 1986). Thereafter, no change in placental dry weight occurs, although placental wet weight may decrease 15 – 20% by term (d 145 ± 2) due to dehydration, loss of glycosaminoglycans, and extensive tissue remodelling (Bell et al., 1999; Ehrhardt and Bell, 1995; Hay, 1996; Molina et
al., 1991). By term, ovine placental cotyledons account for only one tenth of fetal weight (Battaglia and Meschia, 1986). In humans, placental weight increases throughout gestation, albeit at a much slower rate from mid gestation to term (Molteni et al., 1978; Schneider, 1996). This species-specific difference in placental growth patterns is reflected in the difference in fetal:placental weight ratio at term. In sheep this ratio is >10 (Molina et al., 1991) whereas in humans it is between 7 and 9 (Molteni et al., 1978; Schneider, 1996).

In sheep, placental weight (---) peaks at mid gestation and may decrease 15 – 20% by term (d 145 ± 2). In contrast, fetal weight ( — ) gain is most rapid in the second half of gestation. Placental glucose transfer (---) increases over the second half of gestation, reflecting the increasing glucose requirements of the growing fetus (Hay, 1996).

In contrast, early fetal weight gain is slow, followed by a steep increase that may (human) or may not (sheep) flatten towards term (Owens and Robinson, 1988). Approximately 80% of fetal growth occurs in the third trimester (Battaglia and Meschia, 1986; Schneider, 1996). In sheep, fetal weight does not surpass placental weight until after d 90 of gestation (Ehhrhardt and Bell, 1995), but then rapidly outstrips placental weight. There is a greater than 16-fold increase in fetal weight from mid (d 103) to late gestation (d 131.5) (Molina et al., 1991).

1.2.3 Placental nutrient transfer capacity

Placental size and functional capacity are two important determinants of placental nutrient transfer capacity (Hay and Wilkening, 1994).
1.2.3.1 Placental size

Both human and animal studies have demonstrated that fetal weight at term is directly related to placental weight, and studies in sheep support the concept that placental size is an important determinant of fetal growth (Dawes, 1968; Gluckman and Heyman, 1996; Harding et al., 1985; Molteni et al., 1978; Schneider, 1996). Experimental caruncalectomy, in which placental growth is limited by surgical removal of potential placental implantation sites (caruncles) prior to pregnancy, results in reduced placental size, umbilical blood flow, placental to fetal transfer rates of glucose and oxygen, and fetal weight. Although fetal weight is reduced, the fetal:placental weight ratio increases, suggesting that an increase in placental functional capacity may partially compensate for a reduction in placental size (Owens et al., 1987a; Owens et al., 1987b; Owens et al., 1987c). Such studies demonstrate the importance of the third and final phase of placental development, namely placental functional maturation.

1.2.3.2 Placental remodelling and functional maturation

During the second half of gestation, a number of physiological changes occur to increase nutrient transfer function (Table 1.1). Studies using light microscopy have shown villous surface area increases, and that the thickness of the placental membranes decreases throughout gestation (Battaglia and Meschia, 1986; Baur, 1977; Hay and Wilkening, 1994; Owens, 1991; Schneider, 1996). In humans, the maternal-facing syncytiotrophoblast layer expands rapidly, becoming approximately sixfold larger than the fetal-facing cytotrophoblast membrane (Mayhew and Simpson, 1994; Teasdale and Jean-Jacques, 1985). In sheep, the clearance of water or antipyrine (a measure of placental permeability) increases eightfold between mid and late gestation (Bell et al., 1986).

Concomitant with changes in placental membranes are changes in placental hemodynamics. Molina et al. (1991) found at d 76 of gestation (term d 145 ± 2) the uteroplacenta was perfused approximately eight times more rapidly by maternal than fetal blood and consumed approximately five times as much oxygen as the fetus. In the subsequent two months, umbilical blood flow increased more rapidly than uterine blood flow, so that by d 132 the uterine to umbilical blood flow ratio had fallen from 8:1 to 2:1, and fetal oxygen consumption had grown to exceed uteroplacental oxygen consumption by 60%. Uterine blood flow doubled and umbilical blood flow increased approximately eightfold from mid to late gestation (Molina et al., 1991).
## Chapter 1

**Table 1.1** Factors affecting placental nutrient transfer function (Gluckman and Heyman, 1996; Owens, 1991; Schneider, 1996)

<table>
<thead>
<tr>
<th>Uteroplacental and umbilical blood flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane permeability</td>
</tr>
<tr>
<td>surface area</td>
</tr>
<tr>
<td>membrane thickness/diffusion distance</td>
</tr>
<tr>
<td>pore size and density</td>
</tr>
<tr>
<td>Placental metabolism</td>
</tr>
<tr>
<td>Substrate concentration gradients</td>
</tr>
<tr>
<td>Carrier mediated transport</td>
</tr>
<tr>
<td>location</td>
</tr>
<tr>
<td>regulation</td>
</tr>
<tr>
<td>substrate affinity (measured by $K_m$)</td>
</tr>
<tr>
<td>transport capacity (measured by $V_{max}$)</td>
</tr>
</tbody>
</table>

In addition to these physiological changes, late gestation placental nutrient transfer function may be enhanced by increased maternal-fetal concentration gradients, or by maturation of placental transport systems. Measurement of placental glucose transport capacity (PGT) has suggested that both may be important during placental development. Studies in sheep have shown that PGT increases approximately 10-fold over the second half of gestation (Molina et al., 1991). Part of the increase in PGT is due to the increase in maternal-fetal glucose concentration gradient as a result of increased placental and fetal glucose consumption. However, Molina et al. (1991) demonstrated that approximately 60 per cent of the increase in PGT is due to an increase in transport capacity *per se*. Using pregnant sheep they demonstrated a greater than eightfold increase in the maximal velocity ($V_{max}$) of glucose transport over the second half of gestation. $K_m$ ($V_{max}/2$) did not change, suggesting that the increase in PGT was due to an increase in number, and not activity, of GLUTs in sheep placentae (Molina et al., 1991). This study intimates that developmental regulation of placental GLUT concentration plays an important role in the maturation of placental glucose transfer function in late gestation. However, it remains unclear whether this is due to an increase in GLUT concentration per unit of membrane surface area, or to the increase in membrane surface area that occurs in late gestation (Hay and Wilkening, 1994).
1.3 PLACENTAL GLUCOSE TRANSFER

The direction and magnitude of glucose transfer across the placenta depends on transplacental glucose concentration gradients, and the number and activity of GLUT proteins present in placental membranes. Maternal and fetal arterial glucose concentrations, and hence transplacental glucose concentration gradients, are influenced by a number of factors extrinsic to the placenta. In addition, placental endocrine factors and metabolism influence both maternal and fetal arterial glucose concentrations, and thus influence transplacental glucose flux. This section outlines some of the factors that determine placental glucose transfer rate, including maternal nutrient availability, hormonal and metabolic status of both mother and fetus, and placental metabolism and transfer.

1.3.1 Placental glucose transfer model

Widdas (1952) first modelled the process of placental glucose transfer (PGT) based on the assumption that the placenta acts as a simple diffusion membrane, and does not consume glucose. However, perfusion studies using human placenta (Hauguel et al., 1983) and in vivo studies in sheep (Meschia et al., 1980) have demonstrated that late gestation placental glucose consumption may account for 50 – 70% of uterine glucose uptake. Accordingly, Widdas' equation has been modified to include a correction factor \( q'_p \) to account for placental glucose consumption (Simmons et al., 1979). The following equation describes the model, where \( V_{\text{max}} \) represents the maximal flux of glucose and \( K_m \) (Michaelis Menton constant) is the concentration of glucose in the maternal arterial plasma \( (G_A) \) at which the transport mechanisms are half saturated \( (V_{\text{max}}/2) \). \( G_a \) is the concentration of glucose in the fetal arterial plasma, and \( q'_p \) is the net placental glucose consumption from the fetal glucose pool when maternal and fetal arterial plasma glucose concentrations are equal.

\[
\text{PGT} = V_{\text{max}} \left( \frac{G_A - G_a}{G_A + K_m - G_a} \right) - q'_p
\]

As the equation demonstrates, changes in either maternal \( (G_A) \) or fetal \( (G_a) \) glucose concentration will affect glucose concentration gradients and transport, and affect uterine uptake and glucose consumption (Hay and Wilkening, 1994). Under normal conditions, fetal arterial plasma glucose concentration is directly related to maternal plasma glucose concentration (Hay and Meznarich, 1989; Hay et al., 1983). However, Hay and colleagues (Hay, 1995a) have demonstrated independent effects of maternal \( (G_A) \) and fetal \( (G_a) \) arterial
glucose concentrations on placental glucose metabolism and transfer. Using glucose clamp techniques in term pregnant ewes they have shown that maternal glucose concentration determines the rate at which glucose enters the uterus, uteroplacenta and fetus from the maternal circulation (Hay et al., 1984a). Independent changes in fetal arterial glucose concentration determine the proportion of net uterine glucose uptake consumed by the uteroplacenta (Hay et al., 1990) (see Figure 1.4).

![Figure 1.4 Illustration of the reciprocal relationship between placental glucose transfer and uteroplacental glucose consumption that is determined by fetal arterial glucose concentration](image)

1.3.2 Maternal arterial glucose concentration and placental glucose transfer

Maternal factors influence placental glucose transfer by determining maternal plasma glucose concentrations and availability of glucose to the uteroplacenta. They may be defined as components of the external or internal maternal environmental. The external environment determines nutrient substrate availability and maternal intake (eg, food availability, oxygen at altitude). The internal maternal environment determines partitioning of nutrients between mother and conceptus. It is determined by maternal phenotype and influenced by maternal health, metabolism and endocrine status. The maternal endocrine milieu changes with nutrient availability and stage of pregnancy, and may be dysregulated by disease (eg, diabetes mellitus) (Gluckman and Liggins, 1984).
1.3.2.1 Metabolism and maternal nutrient availability

Human

Homeostatic regulation of carbohydrate metabolism involves maintenance of blood concentrations of metabolites (e.g., glucose, fatty acids and amino acids) in the face of variable nutrient intake and tissue utilisation through hormonal control of glucose uptake and hepatic glucose production. Carbohydrate homeostasis is primarily regulated by pancreatic endocrine hormones. Insulin (known as the hormone of abundance) is secreted by the β cells of the pancreatic islets of Langerhans in response to high blood glucose concentrations. It is anabolic, causing increased storage of glucose, fatty acids and amino acids. In contrast, glucagon (secreted by islet A cells) is catabolic, and mobilizes stored glucose, fatty acids, and amino acids from tissues into the bloodstream. Thus, the opposing actions of these hormones maintain relatively constant blood nutrient levels by promoting short-term storage of nutrients following food intake and mobilisation of those nutrients during the post-absorptive period.

Many other hormones have important roles in regulation of carbohydrate metabolism including somatostatin, growth hormone (GH), insulin-like growth factors (IGFs), glucocorticoids, catecholamines, and thyroid hormones. The carbohydrate regulatory effects of IGF-1 will be described later in the context of fetal nutrient utilisation and growth (section 1.3.4). Excellent reviews of homeostatic regulation of carbohydrate metabolism are available in many physiology and biochemistry textbooks, so this topic will not be described in detail in this thesis (Berne and Levy, 1993; Davies and Shaffer Littlewood, 1979; Ganong, 1987; Gluckman and Heyman, 1996; Mathews and van Holde, 1990; Stryer, 1988).

Ruminant

Adult ruminant carbohydrate metabolism differs from that in humans. Ruminants rely predominantly on microbial fermentation in the rumen to break down the dietary carbohydrates found in a herbivorous diet (i.e., cellulose, hemicellulose, pectin, starch). Acetate, propionate and butyrate are the principle short-chain or volatile fatty acids (VFAs) produced by this process. These microbial waste products may represent over 70% of the host ruminant’s caloric intake (Brockman, 1993), and since most hexose and pentose products of polysaccharide degradation are consumed during the pre-gastric fermentation process, the absorbance of glucose from ruminant gut is minimal (Bergman et al., 1970).

Ruminants, therefore, depend on continuous gluconeogenesis to support the normal glucose requirements of tissues that are obligate glucose consumers (e.g., brain), and the additional
glucose requirements of the ewe and conceptus during pregnancy. The VFA propionate is the most important substrate for gluconeogenesis in fed animals, accounting for about half of the glucose synthesis, with acetate, butyrate, lactate, amino acids and other glucogenic precursors making up the balance. The ruminant liver produces up to 85 – 90% of whole-body glucose turnover in animals on a roughage diet, with kidney and gut releasing small amounts of glucose into the circulation. Despite the high rate of glucose production by ruminant liver, the physiological range of glycemia in pregnant ruminants is low (1 – 4 mmol/L) (Bell, 1993) compared to the normal fasting level of glucose in human peripheral venous blood (3.9 – 5.6 mmol/L) (Ganong, 1987). Because of the central importance of maternal glucose supply to fetal metabolism and growth, it is possible that the ovine placenta has adaptations that allow it to provide sufficient glucose to the fetus despite the unique intermediary metabolism and low glucose concentrations of the ovine mother.

1.3.2.2 Metabolic adaptation to pregnancy

During pregnancy, homeostatic regulation of carbohydrate metabolism is overridden by homeorhetic regulation. The Greek derivation of homeorhesis means ‘uniform flow’, and Bauman and Currie (1980) defined homeorhesis as “the coordinated changes in metabolism in various tissues to support a physiological state”. They were the first to apply homeorhesis to the coordinated adaptations in maternal metabolism that promote nutrient partitioning to support growth of the conceptus and development of the mammary glands prior to and during lactation.

During gestation, maternal physiology and carbohydrate metabolism are altered by the addition of a new endocrine organ, the placenta (Liggins, 1994). The placental trophoblast cells secrete peptide and steroid hormones (e.g., human chorionic gonadotropin (hCG), human placental lactogen (hPL) or human chorionic somatomammotropin (hCS), estrogen, and progesterone) that override normal endocrine regulation of maternal metabolism. Although the placental trophoblast cells are of fetal origin, the hormones they produce are largely secreted into the maternal circulation. In this way the fetus may alter the maternal endocrine milieu to promote nutrient partitioning to the conceptus (Bauman and Currie, 1980; Bocking, 1994; Freinkel and Metzger, 1978; Gluckman and Heyman, 1996; Liggins, 1994).

In humans, homeorhetic changes in maternal carbohydrate metabolism in early pregnancy are primarily directed towards promoting maternal storage of nutrients in preparation for growth of the conceptus (Bocking, 1994). Initially the corpus luteum, and later the placenta, increase
maternal concentrations of estrogen and progesterone, giving rise to pancreatic β cell hyperplasia and increased insulin secretion. As a consequence, tissue glycogen deposition and peripheral glucose utilisation are increased, and hepatic glucose production and plasma glucose concentrations are decreased (Bocking, 1994; Liggins, 1994).

As pregnancy progresses, there is a rise in placental production of progesterone, estrogen, and the growth hormone-like hCS (human placental lactogen, hPL) (Bocking, 1994; Freinkel and Metzger, 1978). These hormones have both pro- and contra-insulin effects. They enhance insulin secretion in response to glucose challenge, but they also increase circulating levels of insulin counter-regulatory hormones such as glucagons and cortisol, and promote lipolysis and gluconeogenesis. In humans, these changing hormone levels cause fluctuations in maternal carbohydrate metabolism around meals times to become more exaggerated with advancing gestation. In a seminal paper describing maternal fuel economy during pregnancy, Freinkel (1978) coined the phrase “accelerated starvation (whenever food is withheld) alternating with facilitated anabolism (when food is ingested)” to describe the heightened metabolic oscillations that occur between the fed and fasted state. The exaggerated lowering of blood glucose in the fasted state leads to activation of hepatic gluconeogenesis and rapid mobilisation of fats. Triglycerides, free fatty acids (FFA), glycerol and ketones provide alternate maternal fuels, ‘sparing’ glucose for transplacental flux.

The net effect of these changes in the maternal endocrine milieu is to promote a state of increased peripheral insulin resistance. Consequently, decreased peripheral glucose utilisation and increased mobilisation of stored glucose creates a mildly diabetogenic state in the mother during late gestation, and increases the supply of maternal blood glucose available for transfer to the feto-placental unit (Freinkel and Metzger, 1978).

1.3.2.3 Pathophysiologies: nutritional intrauterine growth restriction (IUGR)

In functional terms, fetal growth restriction occurs in individuals that have been restrained from achieving their genetic potential for growth (Robinson and Owens, 1996). A wide variety of factors may cause intrauterine growth restriction (IUGR) including genetic or toxic factors. However, the most common cause of IUGR is a reduction in nutrient or oxygen supply to the fetus (Gluckman and Harding, 1997).

IUGR fetuses are small for gestational age (SGA) and are generally hypoxemic and hypoglycemic (Owens et al., 1989). They have elevated umbilical lactate levels and reduced
amino acid plasma concentrations (particularly essential amino acids, such as lysine and leucine) (Economides et al., 1991; Nicolaides et al., 1989). IUGR fetuses have elevated plasma concentrations of cortisol, adrenocorticotropic hormone, catecholamines and glucagon, and low circulating levels of insulin, IGF-1 and thyroid hormones (Owens, 1991; Owens et al., 1989).

IUGR is associated with increased rates of perinatal mortality and morbidity (Robinson and Owens, 1996). Neonatal complications include birth asphyxia, meconium aspiration, persistent fetal circulation, necrotising enterocolitis, hypothermia, hypoglycemia, hypocalcemia, and polycythemia (Lapillonne et al., 1997). Beyond these early effects, epidemiological evidence is accumulating to suggest that restricted fetal growth during critical periods of development may have long-term sequelae for adult health (Barker, 1990; Barker, 1999; Barker et al., 1993b).

The timing, duration and nature of the nutritional insult determines phenotype and postnatal consequences. IUGR neonates are usually classified as exhibiting either symmetrical or asymmetrical growth restriction (Barker, 1994). In general, symmetrical growth restriction reflects nutrient deficiency from early in gestation and indicates that growth of both brain and body have been restricted. These infants are likely to remain shorter and lighter than normal infants, and have the poorest mental development. Asymmetrical growth restriction reflects nutrient deficiency during the second and third trimesters, and indicates that although body growth was retarded, brain growth was spared. Infants with asymmetrical IUGR are more likely to exhibit catch-up growth in the first few months of life, reaching values similar to those of average for gestational age (AGA) infants by 1 or 2 years of age (Barker, 1995; Barker et al., 1993a; Godfrey and Barker, 1995). The studies described in this thesis focus on late gestation nutrient restriction.

**Fetal adaptation to reduced maternal nutrient supply**

*In vivo* studies in sheep have shown that sustained fetal hypoglycemia or hypoinsulinemia lead to development of fetal gluconeogenesis, with increased reliance on alternate energy sources, and reduced rates of protein synthesis and growth (Bell et al., 1999; Harding and Johnston, 1995; Milley, 1993). In the short term, fetal oxygen consumption is sustained (Hay et al., 1989), and there is an increase in the breakdown and oxidation of endogenous substrates such as glycogen, lactate, fructose (in ungulates), amino acids, fatty acids and ketoacids (Harding and Johnston, 1995; Hay, 1995a). In the face of chronic reduction in
nutrient supply the fetus decreases glucose utilisation (Hay et al., 1983), and increased production of fetal urea indicates that catabolism of fetal protein (fetal 'wasting') maintains oxidative metabolism (Harding and Johnston, 1995; Hay, 1999; Liechty et al., 1992; van Veen et al., 1987). Protein oxidation may account for up to 80% of fetal oxygen consumption, and protein synthesis is inhibited as amino acid supply is redirected from growth into oxidative metabolism (Simmons et al., 1974).

The redirection of available nutrients into oxidative metabolism aids survival, but results in a slowing or cessation of growth. In sheep, surgically implanted growth measuring devices have demonstrated that late gestation fetal growth slows within 2 – 3 days of the onset of maternal undernutrition (Mellor and Matheson, 1979). When fetuses were exposed to undernutrition for periods of between 7 and 16 days fetal growth generally resumed when nutrient levels were restored. However, no recovery in growth rate occurred if the period of undernutrition was extended past 21 days (Mellor and Murray, 1982).

**Placental adaptation to reduced maternal nutrient supply**

The placenta also adapts to reduced maternal nutrient supply by redirecting available glucose into oxidative metabolism, utilising alternate fuels, and reducing growth rate (Harding and Johnston, 1995; Hay, 1996). Uterine glucose supply regulates partitioning of uteroplacental glucose metabolism into oxidative (CO₂ production) and non-oxidative (lactate and fructose production) pathways (Aldoretta and Hay, 1999). Reduced glucose supply leads to a reduction in placental lactate and fructose production (non-oxidative metabolic products), allowing glucose carbon to be used preferentially for oxidative metabolism (Aldoretta and Hay, 1999; Meznarich et al., 1987). Although glucose oxidation accounts for a smaller fraction of uteroplacental oxygen consumption, uteroplacental oxygen consumption rate does not change (Carver and Hay, 1995; Hay et al., 1990; McGowan et al., 1995a), indicating that other substrates substitute for glucose to maintain oxidative metabolism (Aldoretta and Hay, 1999; Hay, 1995b). During short-term maternal undernutrition, amino acid oxidation may partly substitute for a reduction in placental glucose oxidation (Liechty et al., 1991). Other oxidisable substrates include ketones (Carver and Hay, 1995), acetate and carbon derived from placental carbohydrate and lipid stores (Bell et al., 1999).

With chronic maternal undernutrition/hypoglycemia two additional adaptations occur. Firstly, as fetal gluconeogenesis develops, increased fetal blood glucose concentrations shift the balance of glucose uptake by the uteroplacenta more to placental glucose consumption and
less to glucose transfer to the fetus (DiGiacomo and Hay, 1990a) (Figure 1.4). Secondly, both placental (Aldoretta et al., 1994; DiGiacomo and Hay, 1990b) and fetal (Harding and Johnston, 1995; Mellor and Matheson, 1979) growth rates are reduced. In contrast to the fetus, which shows irreversible changes in growth after chronic maternal undernutrition (Mellor and Murray, 1982), placental lactate and fructose production resume and placental glucose metabolism is restored once nutrient supply is resumed (Aldoretta and Hay, 1999; Harding and Johnston, 1995).

Evidence for regulation of placental GLUTs by reduced maternal glucose supply

Although placental GLUT expression has been shown to be altered by restricted maternal nutrition, maternal hypoglycemia, and uteroplacental blood supply, the results are contradictory. In humans, measurement of GLUT1 protein levels in microvillous (MVM) and basal (BM) membrane fractions showed no difference in GLUT1 expression in placentae from term or pre-term IUGR fetuses compared to normal fetuses (Jansson et al., 1993).

In rats, Reid et al. (1999) induced IUGR by bilateral uterine ligation at d 19 of gestation (term d 21.5) and found GLUT1 and GLUT3 gene expression and protein levels were unchanged on d 20 and 21. Conversely, Das et al. (1998), using a similar model, demonstrated a 50% decrease in GLUT1 protein in d 21 rat placenta.

In sheep, insulin induced chronic hypoglycemia in late gestation has been shown to cause an approximately 30% decline in placental GLUT1 protein levels (Das et al., 1998). Conversely, two weeks restricted maternal energy intake (60% of predicted requirements) resulted in an approximately 20% increase in GLUT3 protein, but no change in GLUT1 protein levels (Bell et al., 1999).

The use of different experimental models makes interpretation difficult, and the evidence is equivocal. Thus far, the effects of reduced maternal glucose availability on placental GLUT expression remain unclear.

1.3.2.4 Pathophysiology: diabetes mellitus

Diabetes mellitus is an endocrine disease that produces a disturbance in carbohydrate homeostasis (Gillmer et al., 1984). Pregnancy is complicated by two types of diabetes; pregestational insulin dependent diabetes mellitus (IDDM) and gestational diabetes mellitus (GDM).
Prior to the introduction of insulin therapy, pregnancy rates were low, and fetal and maternal mortality rates high in pre-gestational diabetics. The advent of insulin therapy in 1922 saw a decline in maternal mortality (Gillmer et al., 1984). However, the incidence of fetal perinatal mortality and congenital malformation remained high. Mills et al. (1979) demonstrated that all common congenital malformations associated with pre-gestational diabetes occur before the seventh week of gestation, and are therefore often established before there is an awareness of pregnancy. This evidence established the importance of strict diabetic control in the periconceptual and early stages of gestation.

Gestational diabetes mellitus (GDM) effects 1 – 3 % of all pregnancies (Freinkel and Metzger, 1978) and is defined as chemical diabetes diagnosed during pregnancy. GDM occurs when the mother can secrete insulin, but not in sufficient quantities to meet the enhanced requirements for insulin that occur in late pregnancy. It may be treated by diet alone (White’s class A1) or with diet and drugs (White’s class A2, Table 1.2).

<table>
<thead>
<tr>
<th>White’s Class</th>
<th>Age of onset (years)</th>
<th>Duration (years)</th>
<th>Retinopathy</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>diagnosed during pregnancy (diet alone)</td>
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<td></td>
</tr>
<tr>
<td>A2</td>
<td>diagnosed during pregnancy (diet and drugs)</td>
<td></td>
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</tr>
<tr>
<td>B</td>
<td>≥ 20</td>
<td>&lt; 10</td>
<td>absent</td>
</tr>
<tr>
<td>C</td>
<td>10 – 19</td>
<td>10 – 19</td>
<td>absent</td>
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<tr>
<td>D</td>
<td>&lt; 10</td>
<td>≥20</td>
<td>present</td>
</tr>
<tr>
<td>F</td>
<td>Nephropathy and/or proliferative retinopathy</td>
<td></td>
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Infants of diabetic mothers (IDM) form a very heterogenous group, depending on the severity of maternal diabetes and the degree of metabolic control achieved during pregnancy. One extreme is represented by the IUGR baby born to mothers that have pre-existing IDDM with severe diabetic angiopathy (Whites class F). In these babies energy stores of fat and glycogen are low and cell size and number are reduced in many organs (Gillmer et al., 1984). This outcome has become more rare with improved clinical management of diabetic pregnancy.

The other extreme is represented by the plethoric, macrosomic infant born to mothers with diabetes of short duration (White’s class B) or pregnancy onset GDM (White’s class A1/A2).
These babies have increased total body protein, glycogen and fat. Pedersen's classical 'hyperglycemia-hyperinsulinism' hypothesis proposed that diminished maternal insulin caused diminished glucose utilisation in the mother (Pedersen et al., 1954). The subsequent rise in maternal glucose leads to a concomitant increase in fetal glucose, thus stimulating fetal insulin production, glucose utilisation and greater fetal growth. Freinkel and colleagues modified the hypothesis to include the stimulatory effects on fetal growth of other substrates in the mother after demonstrating that diabetics have disturbances in all major insulin-dependent nutrient substrates (glucose, FFA, triglycerides, and amino acids) (Metzger et al., 1980). Neonatal complications associated with diabetes include congenital abnormalities, respiratory distress, cardiomegaly, polycythemia, renal vein thrombosis, hyperbilirubinemia, hypocalcemia, hypoglycemia, birth trauma, and feeding problems (Gillmer et al., 1984).

Fetal adaptation to increased maternal glucose supply

In vivo studies in rats and sheep have demonstrated that selective induction of maternal hyperglycemia by maternal glucose infusion increases transplacental flux of glucose and induces fetal hyperglycemia (Hay, 1994; Thomas et al., 1990; Thomas and Lowy, 1992) the length and duration of which determines fetal outcome. Short-term or pulsatile increases in maternal and fetal hyperglycemia stimulate fetal insulin release and promote fetal glucose utilisation (Hay, 1994). Such insulin driven growth may mimic the development of macrosomic babies with gestational diabetes. In contrast, long-term or severe maternal hyperglycemia suppresses fetal insulin release (Carver et al., 1995), and leads to an increase in fetal plasma glucose concentrations. Under these conditions, the transplacental glucose concentration gradient is reduced and excess uterine glucose uptake is diverted into increased placental glucose consumption. Non-oxidative glucose metabolism increases production of carbohydrate storage molecules and placental tissue (eg, lactate and glycogen) (Hay, 1994). Overall, fetal response to chronic hyperglycemia acts to limit fetal growth and adiposity, and may resemble the fetal growth restriction seen with severe maternal diabetes.

Placental adaptation to increased maternal glucose supply

In situ perfusion of rat placenta has demonstrated maternal diabetes induced by chemical (streptozotocin, STZ) destruction of insulin releasing β cells increased placental glucose flux (Thomas et al., 1990) and altered placental metabolism to increase glucose utilisation (Thomas and Lowy, 1992). Fetal weight was not increased, but reverse flux of glucose from the fetus to the placenta was observed and placental glycogen deposition and lactate production both increased (Thomas and Lowy, 1992).
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In sheep, selective induction of maternal hyperglycemia by maternal glucose infusion has also demonstrated that acute maternal hyperglycemia produces an increase in uteroplacental glucose uptake and placental glucose transport rate to the fetus (Hay, 1995b). Under chronic conditions, uteroplacental glucose consumption remains high, but fetal glucose uptake returns to normal levels. This is because chronic fetal hyperglycemia eventually down-regulates fetal pancreatic insulin secretion, leading to decreased fetal glucose utilisation and a diminished maternal-fetal glucose concentration gradient (Aldoretta et al., 1994), conditions that favour glucose flux from fetus to placenta (Figure 1.4).

Uteroplacental oxygen consumption does not change with increased placental glucose consumption (Aldoretta et al., 1994; Hay and Meznarich, 1989), indicating that uteroplacental glucose consumption is increasingly partitioned into non-oxidative pathways (Aldoretta and Hay, 1999). However, the fate of glucose carbon under these conditions is not known. Placental size is not dramatically increased, suggesting that placental glucose carbon surplus to requirements for oxidative metabolism is exported as lactate and fructose rather than being stored in the placenta (Aldoretta et al., 1994; Hay, 1995a). Experiments involving acute maternal hyperglycemia have shown increased uteroplacental lactate production that is partitioned more into the uterine than umbilical circulation (Aldoretta and Hay, 1999).

Evidence for regulation of placental GLUTs by increased maternal glucose supply

In general, in vitro experiments in cultured trophoblast cells have shown negative regulation of GLUT1 by glucose (Klip et al., 1994). Absence of glucose (0 mM) induces GLUT1 protein expression in cultured human syncytiotrophoblasts (Illsley et al., 1998), and high glucose concentrations (> 20 mM glucose) decrease GLUT1 mRNA and protein expression in human (Hahn et al., 1998b; Hauguel-de Mouzon et al., 1994) and rat trophoblast cells (Das et al., 1998). At more physiological levels (1 – 15 mM), GLUT1 protein expression remains remarkably unaffected by increased extracellular glucose levels. In contrast, glucose transporter activity is inversely related to extracellular glucose concentration over the range 1 – 25 mM (Hahn et al., 1998a; Illsley et al., 1998). This discrepancy suggests post-translational regulation by extracellular glucose after GLUT1 has been incorporated into the plasma membrane (Illsley et al., 1998). Hahn et al. (2000) have used immunogold labelling to demonstrate internalisation of cell surface GLUT1 transporters in response to 25 mM extracellular glucose.
In rats, STZ induced insulinopenic diabetes caused no measurable change in GLUT1 mRNA (Boileau et al., 1995) or protein (Boileau et al., 1995; Das et al., 1998) expression in late gestation placentae. In contrast, placental GLUT3 mRNA and protein expression was increased four- to fivefold compared to non-diabetic rats. A concomitant fivefold increase in placental 2-deoxyglucose uptake and glycogen concentration were also observed, indicating that fetal-facing GLUT3 plays an important role in placental glucose metabolism (Boileau et al., 1995).

Furthermore, placental GLUT3 expression responded to subsequent experimental manipulation of maternal glycemia. A five day phlorizin infusion (inhibits renal tubular glucose reabsorption) resulted in a significant decrease in maternal plasma glucose levels in insulinopenic STZ diabetic rats (440 ± 11 to 248 ± 23 mg/dl; normal 89 ± 4 mg/dl) and 2.5- to 3-fold decrease in GLUT3 mRNA and protein levels. Short-term induced hyperglycemia (12 h) induced a fourfold increase in placental GLUT3 mRNA and protein with no concomitant change in placental GLUT1 expression (Boileau et al., 1995). These results indicate that in rats maternal glycemia may play an important role in regulating placental GLUT3 expression.

In humans, GLUT1 is the major glucose transporter present in placental tissue (Illsley, 2000a). GLUT1 is primarily localised to the syncytiotrophoblast, with a greater degree of expression on the maternal-facing MVM compared to the fetal-facing BM (Barros et al., 1995; Jansson et al., 1995; Takata et al., 1992). The asymmetric distribution of GLUT1, coupled with the greater surface area of the MVM (≥ 5-fold), ensures a much higher GLUT1 concentration on the maternal facing MVM than the fetal facing BM (Teasdale and Jean-Jacques, 1985). Such an arrangement maintains intrasyncytial glucose concentrations at levels close to that of maternal plasma, thus maximising the glucose gradient between placenta and fetus. Illsley and colleagues have hypothesised that BM glucose transporters act as the rate-limiting step in trans-syncytiial glucose transport and that, under conditions of normal placental blood flow, basal transporters act as the rate-limiting step in human transplacental transport. As a consequence, they have proposed that changes in basal membrane GLUT1 expression/activity will have substantial effects on transplacental glucose transport (Illsley, 2000a).

Using purified MVM and BM from term pre-gestational (White’s class B) and gestational (White’s class A1/A2) diabetics, they have recently demonstrated significant increases (~2-fold) in the expression of BM GLUT1 protein and D-glucose uptake (~40%) by
syncytiotrophoblast basal membranes in both classes of diabetics. MVM GLUT1 protein expression and activity remained unchanged. Glycosylated hemoglobin (HbA1c) measurements were within the normal range indicating good glycemic control for all diabetics in the study. Nevertheless, analysis of birthweights showed 8 out of 25 neonates from diabetic pregnancies were macrosomic (> 90th percentile) (Gaither et al., 1999). This data is supported by results from a study using the same membrane separation technique to investigate placental GLUT1 in long-term (White’s class D) insulin dependent diabetics (Jansson et al., 1999). BM GLUT1 protein expression and activity were significantly increased (40% and 59% respectively), but MVM expression and activity were unchanged. BM GLUT1 was positively correlated to birthweight, and both birthweight and placental weight were ~20% increased in IDDM compared to normal pregnancies. First trimester HbA1c was significantly increased, but no significant differences were observed in the second and third trimester, indicating optimal maternal glycemic control during the latter stages of pregnancies when fetal growth is greatest.

Illsley and colleagues have hypothesised that BM GLUT1 is involved in the macrosomic growth observed in diabetic pregnancy (Gaither et al., 1999; Illsley, 2000b). They propose that prior to diagnosis or during short periods of inadequate control, maternal hyperglycemia leads to fetal hyperglycemia (see also Pedersen’s hypothesis, section 1.3.2.4). In the fetus, this results in increased production of growth factors such as insulin-like growth factor-1 (IGF-1) that promote feto-placental growth including up-regulation of GLUT1 protein levels in the basal membrane of the syncytiotrophoblast. Such changes create a positive feedback loop, in which elevated expression (and activity) of basal membrane GLUT1 increase fetal hyperglycemia and growth factor production, and lead to continued derangement of the fetal growth axis (Illsley, 2000a; Illsley, 2000b).

In summary, in vitro experiments in cultured trophoblast cells have shown negative regulation of GLUT1 expression by glucose, with absence of glucose (0 mM) inducing and high glucose concentrations (> 20 mM glucose) decreasing GLUT1 expression (Illsley et al., 1998; Klip et al., 1994). In vivo, evidence exists to show regulation of placental GLUTs by reduced maternal nutrient supply, but the data are contradictory (Bell et al., 1999; Das et al., 1998; Jansson et al., 1993; Reid et al., 1999). In contrast, recent evidence in humans (Gaither et al., 1999; Jansson and Lambert, 1999) and rats (Boileau et al., 1995) has clearly demonstrated that expression of fetal-facing GLUT3 (in rats) and BM GLUT1 (in humans) is increased...
when maternal plasma glucose concentrations are elevated by maternal diabetes (Illsley, 2000b).

1.3.3 Placental metabolism and placental glucose transfer

Placental metabolism plays an important role in the provision of nutrients to the fetus. Many of the compounds transferred via the placenta are modified in some way, and the placenta carries out a range of metabolic activities including glycolysis, gluconeogenesis, glycogenesis, triglyceride synthesis, amino acid interconversion, and fatty acid modification (Hay, 1996). This section will describe the metabolism and transfer of major placental metabolites. Placental glucose metabolism has been discussed in section 1.3.2 and will only be briefly mentioned here.

1.3.3.1 Glucose

The placenta is a highly metabolic organ. Under normal conditions, uteroplacental consumption has been shown to account for about 60 – 70% of uterine net glucose uptake in late pregnant ewes (Hay, 1995b). Placental glucose consumption restricts transport of glucose into the umbilical circulation, leading to development of relative fetal hypoglycemia. In this manner, placental glucose consumption plays an important role in maintaining the transplacental glucose concentration gradient (Hay, 1991b).

Tracer studies in pregnant ewes have revealed that about 56% of uteroplacental glucose consumption is rapidly metabolised to lactate (~35%), fructose (~4%), and CO₂ (~17%). These provide an important source of fetal nutrients. It is estimated that fetal oxidation of glucose-derived substrates (eg, lactate, fructose) provides 20% of fetal energy requirements, in addition to the 40 – 50% provided by direct oxidation of glucose (Battaglia and Meschia, 1988; Bell et al., 1999; Hay, 1995b). The metabolic fate of the remaining glucose consumed is unknown (Aldoretta and Hay, 1999; Bell et al., 1999).

1.3.3.2 Oxygen

Oxygen transport across the placenta is flow-limited and occurs by passive diffusion down a concentration gradient. The transplacental PO₂ gradient and placental oxygen permeability are such that placental oxygen transfer is maintained even when the uterine circulation is compromised. A significant reduction in placental oxygen transfer does not occur until uterine
blood flow is reduced to less than 50 percent of normal (Hay, 1996; Wilkening and Meschia, 1989).

Two factors help maintain the transplacental PO₂ gradient. The first is oxygen consumption by the placenta, which has one of the highest rates of oxygen consumption of all tissues in the body. In the late gestation ewe, uteroplacental tissues consume 40 – 50% of oxygen taken up from the uterine circulation (Hay, 1991c). The second factor is the difference in oxygen affinity between maternal and fetal hemoglobin. Fetal hemoglobin has a higher affinity for oxygen than adult hemoglobin. This allows blood oxygen saturation to be higher in fetal blood than maternal blood at the relatively low PO₂ characteristic of uterine venous blood (Doughty and Sibley, 1995; Hay, 1996).

1.3.3.3 Amino acids

Most amino acids are transported into the trophoblast cytosol against their concentration gradients by active (energy dependent) transporters. Currently, at least nine amino acid transporter proteins have been described in the human placenta (Moe, 1995). These are specific for different types of amino acids, and their regulatory influences and location on maternal or fetal membranes have recently been reviewed by Hay (1996, 1998).

At term, the placenta contains a large variety of enzymes capable of metabolising amino acids through metabolic pathways including gluconeogenesis, glycogen synthesis, protein synthesis, amino acid oxidation or modification, and ammonia genesis (Hay, 1991b). In contrast to the high rate of glucose and oxygen utilisation, the late gestation ovine placenta normally consumes only a small proportion of amino acids taken up as an energy source. At this stage of placental development, the fetus is the predominant site for amino acid utilisation (Battaglia, 1986; Battaglia and Meschia, 1978).

Amino acids are important substrates for fetal growth and provide both carbon and nitrogen to the fetus (Battaglia, 1992; Lemons et al., 1976). They are also used as oxidative metabolic fuels (Battaglia and Meschia, 1988). In late gestation fetal sheep (d 120 – 140), amino acid consumption has been estimated to exceed protein synthesis requirement by 2-fold (Jones and Rolph, 1985), and oxidative deamination and fetal ureagenesis account for 25 – 35% of fetal energy requirements (Bell et al., 1999; Silver and Comline, 1976). Tracer studies in sheep have demonstrated that maternal starvation shifts metabolism from sustaining fetal protein synthesis (and growth) towards amino acid oxidation, which contributes significantly towards
maintaining fetal energy needs, and has an important glucose sparing effect (Harding and Johnston, 1995; Mellor and Matheson, 1979).

The placenta cannot synthesise urea (Jones and Rolph, 1985), but produces ammonia that is delivered to both uterine and umbilical circulations over most of gestation (Holzman et al., 1977; Holzman et al., 1979). This provides a valuable source of nitrogen for fetal growth because, unlike urea, ammonia can be reincorporated into amino acids (Jones and Rolph, 1985). Placental-fetal hepatic cycling relationships exist for certain amino acids which contribute to energy and protein balance in the feto-placental unit (eg, glutamine/glutamate, glycine/serine, asparagines/aspartate) (Hay, 1996).

1.3.3.4 Lactate
The mammalian placenta produces large amounts of lactate during aerobic metabolism that enters both maternal and fetal circulations (Sparks et al., 1982). Under hyperglycemic conditions, placental lactate is partitioned about equally into maternal and fetal circulations, whereas under hypoglycemic conditions placental lactate is partitioned more into the fetal than into the uterine circulation (Aldoretta and Hay, 1999; Carver and Hay, 1995). Two factors known to regulate placental lactate production are fetal arterial PO2 and maternal and fetal plasma IGF-1 concentrations (Harding et al., 1994; Liu et al., 1994; Sparks et al., 1983).

The fetus can metabolise lactate with great efficiency under normal conditions of PO2 (Carter et al., 1995). Gluconeogenesis from fetal lactate allows a functional Cori cycle to occur between fetal tissues and the placenta. In the placenta, glucose is reconverted to lactate (and in ungulates fructose) and preferentially released to the umbilical circulation (Sparks et al., 1982). This lactate cycle retains carbohydrate in the fetal compartment and restricts its loss back to maternal circulation (Jones, 1991).

Lactate provides an important carbon source for placental oxidative metabolism, and the ability of the placenta to utilise lactate in oxidative metabolism may spare other substrates (eg, glucose) for fetal oxidative metabolism (Hay and Wilkening, 1994).

1.3.3.5 Fructose
Placental production of fructose is unique to 'fructogenic species' such as ruminants and does not occur to any appreciable extent in humans (Hay and Wilkening, 1994). Its function and metabolic fate are largely unknown. Tracer studies in well-fed normoglycemic pregnant ewes
have shown that placental fructose enters the umbilical vein exclusively, and does not cross the placenta to the maternal circulation (Meznarich et al., 1987). Studies in pregnant sheep have demonstrated a fall in fetal fructose concentrations with prolonged maternal fasting, suggesting it may provide a reservoir of relatively slowly metabolised substrate for glucogenesis that is exclusive to fetal circulation (Hay, 1979).

1.3.3.6 Lipids

The amount and type of lipids transported by the placenta varies among species, being greatest in the hemochorial placenta of human, guinea pig and rabbit, and least in the endotheliochorial placenta of carnivores and the epitheliochorial placenta of ruminants. This species difference is reflected by the fat content of the fetus at term. In humans, adipose tissue may account for as much as 18% of fetal body weight at term. By comparison fetal sheep have limited lipid transport across the placenta, and have little (primarily brown) adipose tissue stored at term (Battaglia and Meschia, 1986).

Placental lipid transport supplies essential fatty acids during placental and fetal growth and development (Hay, 1995a). After entering the placenta, FFA may be transferred directly to the fetus via carrier-mediated transport systems, or may be used by the placenta for triglyceride synthesis, cholesterol esterification, membrane biosynthesis or oxidation (Hay, 1995a). Placental FFA metabolism and transfer is concentration dependent, and placental triglyceride concentrations increase in women who are fasting, who have pre-term infants, or who have diabetes mellitus, all conditions in which maternal plasma FFA concentrations are raised (Freinkel and Metzger, 1978).

Although FFAs are important in adult (especially ruminant) metabolism (Bell, 1993), their role as fuels during fetal life is relatively small. Activity of the enzyme carnitine palmitoyl transferase is low during fetal life, and may account for the limited free fatty acid oxidation in the fetus (Warshaw, 1979).

1.3.4 Fetal arterial glucose concentration and placental glucose transfer

Fetal blood glucose concentration is closely correlated to maternal blood glucose concentration (Economides et al., 1991; Hay et al., 1984b), and excursions in maternal plasma blood glucose levels are rapidly mirrored in fetal blood glucose concentrations, albeit at lower levels. Therefore, the fetus is vulnerable to alterations in maternal blood glucose
levels such as may occur with maternal undernutrition or diabetes, and which may lead to fetal pathophysiologies such as intra-uterine growth restriction (IUGR) or macrosomia. One means by which the fetus may be protected from fluctuations in maternal blood glucose levels is by alterations in placental glucose transfer function. This section describes endocrine regulation of fetal glucose utilisation, and consequent effects on fetal arterial glucose concentration and placental glucose transfer.

1.3.4.1 Endocrine regulation of fetal glucose utilisation

The major nutrient substrates required for fetal growth and development are glucose, lactate, amino acids, water, and oxygen (Battaglia and Meschia, 1986). Under normal conditions, endocrine factors cause nutrients to be taken up from the fetal circulation and utilised as carbon and nitrogen sources for fetal oxidative metabolism and tissue accretion/growth (Battaglia and Meschia, 1988).

Endocrine regulation of growth in the fetus differs from that in the postnatal animal, and is less well understood. In the postnatal animal, growth hormone (GH) is the major growth-promoting hormone, with its actions mediated by insulin-like growth factors (IGF-1 and IGF-2) and their binding proteins (IGFBPs) (Gluckman and Liggins, 1984). In the fetus, the role of GH is unclear. Although circulating levels are high (Gluckman, 1995), GH does not stimulate fetal growth through the same pathways as postnatal growth (Bauer et al., 1998). Rather, experimental and clinical evidence indicates that insulin and IGF-1 are the major growth promoting hormones during late gestation, and their levels are regulated by nutrient availability (Gluckman, 1995; Harding and Johnston, 1995; Oliver et al., 1999).

Insulin plays a permissive role in fetal growth by stimulating glucose uptake into tissues. Over the second half of gestation, fetal insulin concentrations increase (developmentally and in response to secretagogues) (Hay, 1995a), and the fraction of fetal body weight that consists of insulin sensitive tissues (ie, muscle and fat) increases (Bell et al., 1987). As a result, fetal glucose uptake and utilisation increases throughout gestation, lowering fetal arterial glucose concentration, and promoting uteroplacental glucose partitioning to the fetus (Hay, 1996) (Figure 1.4).

Insulin-like Growth Factor-1 (IGF-1) and IGF-2 are peptide growth factors that are structurally and functionally related to insulin (Sara and Hall, 1990). Their effects on
metabolism and growth are thought to be primarily mediated via the IGF-1 receptor (Liu et al., 1993; Sara and Hall, 1990). The IGFs in circulation are reversibly associated with specific, high-affinity binding proteins (IGFBPs) that prolong the half-life of IGFs, and modulate their actions in tissues (Sara and Hall, 1990). To date, six IGFBPs have been identified whose expression varies with tissue of origin, stage of development, and hormonal and nutritional status (Binoux, 1996).

In both humans and sheep, circulating IGF-1 levels correlate with fetal and neonatal size (Gluckman, 1995; Gluckman et al., 1983), and IUGR is associated with reduced fetal IGF-1 concentrations in utero (Lassarre et al., 1991; Owens et al., 1994). In rodents, knockout gene experiments have unequivocally demonstrated that loss of IGF-1 peptide leads to severe hypoplasia of organ and muscle tissue and delays in ossification and development of the central nervous system. Such compromised fetal development results in death of the neonate or in severe growth retardation associated with postnatal mortality (Baker et al., 1993; Liu et al., 1993). Until recently, evidence for the endocrine influence of IGF-1 in large mammals has been largely indirect. However, chronic administration of IGF-1 to fetal sheep has been shown to promote growth of major organs, endocrine glands, and skeletal maturation (Lok et al., 1996), and IGF-1 infusion to the rhesus monkey fetus promotes organ growth and provides beneficial effects to the developing immune system (Tarantal et al., 1997).

Experimental evidence indicates that fetal glucose uptake and utilisation are regulated by a glucose/insulin/IGF-1 axis. Studies in fetal sheep have demonstrated that nutrient availability is the major determinant of fetal IGF-1 levels (Bassett et al., 1990). Maternal undernutrition leads to a decrease in fetal IGF-1 levels, which are restored by glucose or insulin but not by amino acid infusion (Oliver et al., 1993). Fetal pancreatectomy also leads to a reduction in fetal IGF-1 levels and reduced fetal growth (Gluckman et al., 1987). These studies together suggest that circulating fetal glucose levels regulate fetal insulin release, and both regulate fetal IGF-1 production, which ultimately regulates fetal growth.

**Evidence for regulation of placental metabolism and nutrient transfer by fetal IGF-1**

In addition to direct fetal growth promoting effects, IGF-1 also influences the distribution of nutrient supplies. Experimental studies in sheep have shown that short-term infusion of IGF-1 into the fetal circulation enhances fetal glucose and amino acid uptake, and reduces fetal protein breakdown. In addition, placental uptake of amino acids from maternal circulation is increased and placental lactate production inhibited (Harding et al., 1994). These changes not
only demonstrate direct anticatabolic and anabolic effects of fetal IGF-1 on fetal growth, but also indicate that IGF-1 may regulate placental metabolism and nutrient partitioning between placenta and fetus.

**Evidence for regulation of placental GLUT1 and GLUT3 by fetal IGF-1**

Little is known about regulation of GLUT1 and GLUT3 by fetal endocrine factors. However, what evidence is available is consistent with a stimulatory effect of fetal growth hormones on placental GLUTs (Illsley, 2000a). Studies using first trimester trophoblast cells have demonstrated that insulin and hypoglycemia increased GLUT activity and GLUT1 mRNA (Gordon et al., 1995), and that insulin, IGF-1 and IGF-2 enhanced glucose transport in dose dependent fashion (Kniss et al., 1994).

Increases in GLUT1 mRNA and protein and activity in response to insulin have been reported in 3T3 L1 adipocytes (Tordjman et al., 1989) and L6 muscle cells (Taha et al., 1995). GLUT3 has also been shown to be regulated by insulin in cell culture (Taha et al., 1995; Thomas et al., 1996). Although current dogma holds that the placenta is not insulin sensitive (Brunette et al., 1990; Challier et al., 1986; Hahn and Desoye, 1996), insulin may have regulatory effects via the type-1 IGF receptor (Gordon et al., 1995; Illsley, 2000a; Kniss et al., 1994).

In summary, in vivo studies in sheep have demonstrated that fetal IGF-1 may alter placental metabolism to favour glucose partitioning to the fetus (Harding et al., 1994). In vitro studies have shown IGF-1 enhances glucose transport in placental trophoblast cells (Gordon et al., 1995; Kniss et al., 1994). However, to date there is no direct evidence for fetal IGF-1 regulating placental GLUT1 and GLUT3.
1.4 PLACENTAL GLUCOSE TRANSPORTER PROTEINS

Transport of glucose against the concentration gradient, such as occurs across the lumen of the small intestine, occurs via glucose-Na\(^+\) symport proteins (Bell et al., 1990). All other glucose transport occurs by facilitated diffusion; a process by which substrate specific transmembrane transporter proteins facilitate substrate transfer rate many thousandfold over that calculated for simple diffusion (Darnell et al., 1990; Mathews and van Holde, 1990). This section introduces the placental glucose transporter (GLUT) proteins GLUT1 and GLUT3, and describes what is known of their ontogenetic regulation and location during placental development.

1.4.1 General introduction to glucose transporter (GLUT) proteins

Facilitative glucose transport is mediated by a closely related family of transmembrane GLUT proteins. Encoded by six known functional genes and one pseudogene, this family of glycoproteins differs in its affinity for monosaccharides, regulation, and tissue-specific gene expression (Gould and Holman, 1993; Pessin and Bell, 1992). The kinetic property of each GLUT is described by its \(K_m\) (Michaelis Menton constant) value, which is inversely related to its affinity for glucose. The \(K_m\) \(\left(V_{\text{max}}/2\right)\) is equal to the glucose concentration at which the rate of glucose transport is half of its maximal value \(V_{\text{max}}\), or the concentration of glucose at which half the active transporters are occupied in transporting glucose. The \(K_m\) provides both an indication of the propensity of the GLUT to transport glucose, and the level of blood glucose at which the transporter is still able to increase its transport rate.

Analysis of predicted amino acid sequence reveals GLUT1 – 7 are similar in size (-500 aa) and structure (Figure 1.5). Common features include 12 predicted amphipathic helices forming 12 transmembrane domains (TMD), with both amino terminal (N-terminal) and carboxyl (C-terminal) termini located in the cytosol. A large extracellular loop lies between TMD 1 and 2 and contains an asparagine residue that serves as the single site of N-linked glycosylation. A second large intracellular loop between TMD 6 and 7 halves the structure into N-terminal and C-terminal domains. The amino acid sequences joining the remainder of the TMDs form very short loops (-8 aa) which are a conserved feature of the GLUT family. The 12 TMDs share greatest homology between GLUT isoforms, with sequence in the N-terminal and C-terminal regions and the large extracellular loop most divergent. See Gould and Holman (1993), McGowan et al. (1995b) and Pessin and Bell (1992) for reviews.
Figure 1.5 Predicted structure of the mammalian facilitated glucose transporters. The transmembrane domains (TMD) are shown as boxes (1 – 12). A potential site for N-glycosylation in the extracellular loop connecting TMDs 1 and 2 is shown. Non-conserved residues are shown as open circles. Single letter amino acid codes indicate residues that are identical among the known mammalian isoforms. Residues shown in black near the COOH terminal are known to influence transporter function (Bell et al., 1993).

Initially, the members of the GLUT family were named according to the tissue or cell type from which they were isolated. As the number of proteins discovered increased, a uniform nomenclature was developed that numbered the GLUTs in order of their cloning. At first, the tissue distribution of each GLUT was thought to be quite distinct (Table 1.3). However, improved molecular and biochemical detection techniques have allowed more accurate localisation of GLUTs, and it is now apparent that each tissue may contain several different types of GLUT protein. The number, type, and cellular location of each GLUT isoform defines the tissue’s capacity for glucose transport.
Chapter 1

Table 1.3 Tissue distribution of facilitated glucose transporter isoforms (Gould and Holman, 1993; Illsley, 2000a)

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1</td>
<td>Expressed in many fetal and adult tissues; abundant in erythrocytes; blood-tissue barriers including blood-brain barrier, placenta, retina; kidney; adipose and muscle tissue; endothelium; tissue culture cells</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Hepatocytes, small intestine, kidney, pancreatic β-cells</td>
</tr>
<tr>
<td>GLUT3</td>
<td>Neurons; low levels in placenta, kidney, liver small intestine and heart</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Skeletal muscle, heart and adipocytes</td>
</tr>
<tr>
<td>GLUT5</td>
<td>Small intestine (jejunum); lesser amounts in kidney, adipose, muscle and brain</td>
</tr>
<tr>
<td>GLUT6</td>
<td>Pseudogene; not translated into protein</td>
</tr>
<tr>
<td>GLUT7</td>
<td>Liver microsomes</td>
</tr>
</tbody>
</table>

1.4.1.1 Molecular pathways that regulate expression, activation and localisation of GLUTs.

Glucose provides a universal substrate for aerobic and anaerobic metabolism and is of central importance in cellular homeostasis. Therefore, control of glucose transport is an important step in the action of hormones and physiological stimuli that influence cellular metabolism of glucose. GLUT expression and function are isoform and tissue specific, and are regulated by a host of physiological and pathophysiological stimuli and conditions including substrate availability (glucose and glycolytic substrates), serum and growth factors (insulin, insulin-like growth factors, epidermal growth factor, growth hormone and thyroid hormones), cAMP, calcium ionophores, alkaline pH, hypoxia and/or inhibition of oxidative phosphorylation, and oncogenic transformation (For reviews refer Gordon et al., 1995; Illsley, 2000a; McGowan et al., 1995b; Shepherd and Kahn, 1999; Zhang et al., 1999). The following paragraphs review the molecular pathways by which glucose, insulin and insulin-like growth factors, and hypoxia signal alterations in expression, activation and localisation of GLUTs.

Stimulation of glucose transport can be classified as ‘acute’ or ‘chronic’ response. Acute response is mediated entirely by post-translational mechanisms: either by ‘translocation’ of glucose transporters from intracellular storage vesicles to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) or by ‘activation’ of glucose transporters already pre-existing in the plasma membrane (Mueckler et al., 1985; Zhang et al., 1999).
Chronic responses are associated with increased cellular levels of glucose transporter mRNA and/or protein, and may be achieved by enhanced transcription, increased stabilisation of mRNA turnover, and/or decreased turnover of protein (Hahn et al., 2000; McGowan et al., 1995b).

Glucose deprivation causes rapid and sustained increases in cellular GLUT1 mRNA and protein levels (Gordon et al., 1995; Hauguel-de Mouzon et al., 1994). The regulatory mechanisms remain undefined, although glucose may regulate its own uptake independent of insulin. Glucose and/or its metabolites may act via various trans-acting factors to down-regulate GLUT1 mRNA transcription (Klip et al., 1994), and/or regulate glucose transporter biosynthesis via post-transcriptional mechanisms involving either decreased GLUT1 mRNA stability or inhibition of translation (Maher and Harrison, 1990; Boado and Pardridge, 1993).

Insulin and insulin-like growth factors may activate both mitotic and metabolic signalling cascades and stimulate both acute and chronic glucose transport responses. Tyrosine kinase activity of the insulin and insulin-like growth factor receptors leads to the tyrosine phosphorylation of IRS and Shc proteins. The phosphotyrosine residues in these proteins interact via SH2 (Src homology 2) domains with key signalling intermediates including Grb2-SOS and PI 3-kinase (phosphatidylinositol 3-kinase). The Grb2-SOS system leads to activation of the Map kinase pathway via Ras activation (mitotic stimulation leading to cell growth), while the PI 3-kinase pathway leads to metabolic stimulation including glucose transport and glycogen synthesis (Holman and Kasuga, 1997). Downstream targets of PI 3-kinase include protein kinase C isoforms ζ (zeta) and λ (lambda), protein kinases of the Akt/protein kinase B system, and proteins that regulate membrane related functions such as actin assembly, membrane ruffling, and early endosome fusion (Czech and Corvera, 1999; Holman and Kazuga, 1997).

Hypoxia may also induce both acute and chronic stimulation of glucose transport. The acute response to hypoxia is mediated either by translocation of glucose transporters to the plasma membrane (GLUT1 and GLUT4), or by activation of glucose transporters pre-existing in the plasma membrane (GLUT1) (Zhang et al., 1999). However, the same signalling pathway does not mediate translocation of glucose transporter proteins in response to insulin and hypoxia. The effects of insulin and hypoxia on glucose transport are additive, and Wortmannin (an inhibitor of PI 3-kinase) blocks stimulation of glucose transport by insulin, but not hypoxia (Yeh et al., 1995). The chronic response to hypoxia is associated with increased cellular levels of GLUT1 mRNA and protein, and is mediated by dual mechanisms operating in
parallel. A decrease in the concentration of oxygen *per se* upregulates expression of hypoxia inducible factor-1 (HIF-1) transcription factor, which stimulates GLUT gene transcription by binding to specific DNA elements (hypoxia inducible elements - HIE) located in the 5'- and 3'-flanking regions of glucose transporter genes. Inhibition of oxidative phosphorylation secondary to hypoxia is associated with decreased cellular ATP content, and has been shown to have dual effects on GLUT biosynthesis. GLUT1 mRNA transcription is upregulated via the HIF signalling pathway, and GLUT1 mRNA turnover is stabilised by specific RNA binding proteins attaching to AU-rich motifs in the 3'-UTR (Zhang et al., 1999).

### 1.4.2 Placental glucose transporters: GLUT1 and GLUT3

Although recent studies have detected other GLUT isoforms in rat (Zhou and Bondy, 1993) and human (Hauguel-de Mouzon et al., 1994; Reid and Boyd, 1994) placenta, GLUT1 and GLUT3 are the most abundant isoforms in mammalian placenta (Hahn and Desoye, 1996; Illsley, 2000a) and are the focus of this review.

#### 1.4.2.1 GLUT1: erythrocyte-type glucose transporter

GLUT1 protein was first isolated in 1977 from human erythrocytes by Kasahara and Hinkle (Kasahara and Hinkle, 1977). Eight years later Mueckler and Lodish cloned and sequenced GLUT1 from a HepG2 cDNA library using an antibody prepared against the purified erythrocyte protein (Mueckler et al., 1985). Originally termed HepG2/erythrocyte/brain transporter, GLUT1 is a major component of erythrocyte plasma membrane, comprising 3 - 5% of total protein. Subsequently, it has been found to be present in many other cell types and is the principal glucose transporter in fetal tissues (McGowan et al., 1995b; Mueckler et al., 1985). The ubiquitous distribution of GLUT1 suggests it is responsible for constitutive or basal glucose uptake (Pessin and Bell, 1992).

The amino acid sequence of GLUT1 is highly conserved, and there is 97 - 98% identity between the human (Mueckler et al., 1985), rat (Birnbaum et al., 1986), rabbit (Asano et al., 1988), mouse (Kaestner et al., 1989), and bovine (Boado and Pardridge, 1990) sequences. The high degree of sequence conservation suggests all regions of the protein are functionally important (Birnbaum et al., 1986).

GLUT1 is a low affinity glucose transporter with a relatively high \( K_m \) value. GLUT1 shows asymmetric kinetic properties, with \( K_m \) efflux values higher than \( K_m \) influx values. This
means GLUT1 has a lower affinity for transporting glucose out of the cell, and it has been proposed that this asymmetry would allow GLUT1 to act effectively as a unidirectional transporter when extracellular glucose levels are low, and intracellular glucose demand is high. GLUT1 is also capable of transporting galactose (Gould and Holman, 1993), and dehydroascorbic acid/vitamin C (Prasad et al., 1998).

1.4.2.2 GLUT3: brain-type glucose transporter

Bell and co-workers (Kayano et al., 1988) used low stringency conditions to screen a human fetal skeletal muscle cDNA library to isolate GLUT3. It has 64% and 57% amino acid identity with GLUT1 and GLUT4 isoforms respectively (Sargeant et al., 1993) and is not as highly conserved among species as GLUT1 with 83% amino acid sequence identity between human (Kayano et al., 1988) and mouse (Nagamatsu et al., 1992) GLUT3.

The distribution of GLUT3 is more restricted than GLUT1, and this isoform is predominantly expressed in tissues exhibiting high levels of glucose utilisation (McGowan et al., 1995b). Northern blot analysis revealed the predominant site of GLUT3 gene expression in humans to be the brain, with lower levels in placenta, kidney, liver, fat and muscle (Kayano et al., 1988; Nagamatsu et al., 1992). In monkeys, rabbits, rats and mice GLUT3 gene expression is restricted to brain and neural cells (Nagamatsu et al., 1992; Pessin and Bell, 1992).

GLUT3 exhibits a relatively low $K_m$, and therefore high affinity, for glucose. The expression of GLUT1 at the blood-brain barrier and GLUT3 in neural cells suggests that both transporters are required to transport glucose to the brain. It has been proposed that GLUT1 and GLUT3 act in tandem to meet the high energy demands of such tissue, and that the relatively high affinity of GLUT3 for glucose compared to GLUT1 ensures efficient uptake of glucose by neuronal cells even at low extracellular glucose concentrations (Gould and Holman, 1993; Pessin and Bell, 1992).

In human tissue, Northern analysis reveals two GLUT3 transcripts with a major transcript at $\sim$4.1kb and a second transcript at $\sim$2.8kb (Clarson et al., 1997; Fukumoto et al., 1988; Hauguel-de Mouzon et al., 1997; Kayano et al., 1988). Sequence data confirmed the size difference was a consequence of alternative polyadenylation generating mRNAs with 3' untranslated regions (3'UTR) of different lengths. In other species a single GLUT3 mRNA transcript is observed at $\sim$4.1kb (Gerhart et al., 1992; Nagamatsu et al., 1992). A number of studies have confirmed that in rabbit (Asano et al., 1988), rat (Boileau et al., 1995; Zhou and
Bondy, 1993), and sheep (Bennett et al., 1995; Currie et al., 1997) placenta GLUT3 mRNA is present and expressed as a single size transcript of ~4.1 kb.

Despite clear evidence for the presence of GLUT3 mRNA in the placenta of several species, initial reports as to the presence of GLUT3 protein in human placenta were conflicting. Some studies found evidence for its presence (Haber et al., 1993; Shepherd et al., 1992), whereas other studies found none (Barros et al., 1995; Jansson et al., 1993; Maher et al., 1992). More recent studies have demonstrated that although GLUT3 mRNA is present throughout villous tissue (Hauguel-de Mouzon et al., 1997; Jansson et al., 1995), GLUT3 protein expression is restricted to fetal vascular endothelium (Hauguel-de Mouzon et al., 1997) (section 1.4.4).

1.4.3 Ontogeny of GLUT1 and GLUT3

In humans, evidence about ontogenesis of placental GLUTs is equivocal. Hauguel-de Mouzon (1994) reported that GLUT1 mRNA levels were fivefold higher in first trimester (8 – 10 weeks) villous tissue than in term placenta. In direct contrast, Sakata et al. (1995) reported [3H] cytochalasin B binding, GLUT1 mRNA and protein levels all increased (7 – 10, 18 – 20, and 38 – 40 weeks), and GLUT3 mRNA decreased during pregnancy. Jansson et al. (1993) found no significant change in GLUT1 protein levels on MVM, but showed GLUT1 protein increased in BM during pregnancy.

In rats, high levels of GLUT1 mRNA were observed in the junctional zone during mid gestation placental growth, followed by a decrease in expression towards term. The level of labyrinthine GLUT3 mRNA did not change from mid gestation through term. However, the volume of the labyrinth, and consequently total GLUT3 gene expression, increased greatly during this period (Zhou and Bondy, 1993).

In sheep, Northern and Western blots demonstrated that GLUT1 mRNA and protein increased from d 75 – 110, with no further change at d 140, whereas GLUT3 mRNA and protein increase throughout gestation (Ehrhardt and Bell, 1997).

1.4.4 Localisation of GLUT1 and GLUT3

Zhou and Bondy (1993) published the first paper describing the localisation of placental glucose transporters in rat placenta. Using in situ hybridisation they reported that in rat placenta both GLUT1 and GLUT3 were co-localised in the labyrinthine zone (principal site of
materno-fetal exchange of substances), and GLUT1 mRNA alone was present in the placental growth plate or junctional zone (site of trophoblast proliferation during placental expansion that contains only maternal blood vessels). In the labyrinth, three trophoblast cell layers (cytotrophoblast, and syncytiotrophoblast I and II) separate maternal from fetal circulations. Zhou and Bondy (1993) observed that GLUT1 mRNA was clearly present in the (maternal facing) cytotrophoblast as well as syncytiotrophoblast layers, whereas by light microscopy GLUT3 mRNA appeared to be expressed only in the inner (fetal facing) syncytiotrophoblast II. They proposed that the primary function of GLUT1 was to transport glucose from maternal circulation for use as a placental fuel, and that GLUT3 was important for glucose transfer to the fetus.

Boileau et al. (1995) and Shin et al. (1997) confirmed that in rat placenta both GLUT1 and GLUT3 protein are present in the labyrinth, with GLUT1 protein only in the junctional zone. In human placenta, there is consensus as to the presence of GLUT1 protein at both the maternal-facing MVM and the fetal-facing BM of the syncytiotrophoblast layer (Arnott et al., 1994; Barros et al., 1995; Hauguel-de Mouzon et al., 1997; Jansson et al., 1995; Sakata et al., 1995). However, localisation of GLUT3 protein has been controversial, and the discrepancy between reported GLUT3 mRNA expression (Kayano et al., 1988) and protein levels (Hauguel-de Mouzon et al., 1997) in human placenta has emphasised that mRNA abundance cannot be taken as an index of protein concentration.

Shepherd (1992) and Haber (1993) detected low levels of GLUT3 protein in human placental homogenate, but Jansson (1993) and Barros (1995) could detect no GLUT3 protein in purified syncytial membrane preparations. Recently, Hauguel-de Mouzon (1997) clearly established the presence of both GLUT3 mRNA and protein in human term placenta. In situ hybridisation demonstrated a discontinuous distribution of GLUT3 mRNA, with intense signal in the trophoblast layer of some intermediate and terminal villi, and none in adjacent villi. Positive signal was also detected in fetal vascular endothelium. Immunocytochemical analysis revealed GLUT3 immunoreactivity was found almost exclusively in fetal vessel endothelium, with none detected in the trophoblast layer. Interestingly, although two GLUT3 transcripts of 4.1 kb and 2.7 kb were detected by Northern blot in both whole placenta and endothelial cells, endothelial cells predominantly expressed the smaller 2.7 kb transcript which is known to be generated by alternative polyadenylation (Kayano et al., 1988). This data may provide the first clue to the puzzling discrepancy between GLUT3 gene expression and protein levels since current evidence indicates that the poly (A) tail may be involved in the regulation of
mRNA decay and translation initiation (McGowan et al., 1995b). In addition, Western blot analysis showed GLUT3 protein had a higher apparent molecular mass in endothelial cells than in placental membranes (~52 vs 49 kDa), suggesting alternate glycosylation of the two fractions (Hauguel-de Mouzon et al., 1997; Illsley, 2000a).

The expression of GLUT1 and GLUT3 mRNA and protein have been investigated using in vitro techniques. Clarson et al. (1997) demonstrated GLUT3 mRNA in both JAr choriocarcinoma cells and in cultured primary human trophoblast cells. In contrast, GLUT3 protein, though expressed strongly in the carcinoma cell line, was absent in differentiated trophoblast cells. The authors proposed that expression of GLUT3 protein may be limited to metabolically active mitotic cells, and that GLUT3 may be involved in maintaining metabolic requirements of dividing trophoblast cells, rather than having a direct role in transport of glucose to the fetus.

As the authors of these papers point out, the specialized location of GLUT3 protein either in fetal endothelial cells (Hauguel-de Mouzon et al., 1997) or in dividing trophoblast cells (Clarson et al., 1997) may account for the confusing reports about the presence of GLUT3 protein in human placenta. Studies using crude membranes from total placental homogenate (Haber et al., 1993; Shepherd et al., 1992) have demonstrated low levels of GLUT3 protein, whereas studies using purified MVM and BM membranes prepared from the syncytiotrophoblast have detected none (Barros et al., 1995; Jansson et al., 1993).

In summary, oGLUT1 and oGLUT3 were chosen for study in this thesis based on known tissue distribution of GLUT1 – 7 (Gould and Holman, 1993). Northern and Western data have demonstrated that developmental regulation of human placental GLUTs differ from other mammalian species (Hauguel-de Mouzon et al., 1997). In rat placenta, the different temporal patterns of GLUT1 and GLUT3 gene expression during placental development lead to the hypothesis that GLUT1 is responsible for maternal to placental glucose transfer, whereas GLUT3 transports glucose between placenta and fetus (Zhou and Bondy, 1993). Testing this hypothesis relies on demonstration of placental GLUT localisation in the syncytiotrophoblast membranes. Although localisation studies in rats have given weight to this hypothesis (Shin et al., 1997; Zhou and Bondy, 1993), thus far no evidence is available for placental GLUT localisation in sheep placenta.
1.5 RESEARCH OBJECTIVES AND THESIS OUTLINE

Glucose transport occurs by facilitated diffusion. The nature of facilitated diffusion implies that transplacental glucose transport may be regulated systemically by factors that alter transplacental glucose concentrations gradients, or at the molecular level by factors that regulate expression and/or activity of placental GLUT proteins. When this project began, there was evidence to support regulation of transplacental glucose transfer by systemic factors that altered the maternal-fetal concentration gradient (Hay, 1991b; Thomas et al., 1990), but little was known about direct molecular regulation of placental GLUTs. Therefore, the overall aim of this thesis was to investigate molecular regulation of placental GLUTs, and the unifying hypothesis for this thesis was:

**Placental GLUT gene expression is regulated during placental development and function.**

Specific hypotheses tested by studies in this thesis were:

**Ovine equivalents of GLUT1 and GLUT3 are present in sheep placenta (Chapter 3).**

In sheep, large size and easy husbandry makes catheterisation of both mother and fetus possible, and allow long-term physiological studies of conscious animals. The ovine model has become a well-characterised model of nutrient transfer during pregnancy, and was selected for these studies because such a model would allow comparison of placental GLUT molecular physiology with metabolic and endocrine data from complementary functional studies *in vivo.*

Two predominant GLUT proteins are expressed in primate and rodent placental tissue: GLUT1 and GLUT3 (Gould and Holman, 1993). Therefore, these isoforms were selected for study in ovine placenta. Because ovine specific cDNAs were not available, the first (and pivotal) aim of the thesis was to isolate, sequence, and characterise ovine GLUT1 and GLUT3 cDNAs.

**Ovine placental GLUT1 and GLUT3 gene expression is regulated during placental development (Chapter 4).**

When these experiments began, no data was available on normal gestational development of placental glucose transporters in sheep. Therefore, the second series of studies focused on characterising developmental regulation and localisation of oGLUT1 and oGLUT3 in ovine placenta at selected time-points from early to late gestation.
Ovine placental GLUT gene expression is regulated by maternal nutrition and glycemia (Chapter 5).

Maternal nutrient levels determine the nutrient supply available to the conceptus, and are known to alter the rate of placental glucose transfer to the fetus (Thomas and Lowy, 1992). The third series of studies was designed to investigate whether global maternal undernutrition or, more specifically, altered maternal plasma glucose concentrations directly regulated placental oGLUT1 and oGLUT3 gene expression.

Ovine placental GLUT gene expression is regulated by fetal IGF-1 (Chapter 6).

Fetal endocrine factors regulate utilisation of the nutrients supplied to the fetus, and normally promote glucose transfer to the fetus by steepening the glucose concentration gradient between mother and fetus (Hay, 1995a). Furthermore, recent evidence has suggested that the fetus may in part direct its own nutrient supply by regulating placental nutrient metabolism, and influencing the partitioning of nutrients between placenta and fetus (Gluckman, 1995). The final set of studies in this thesis investigated whether the fetal growth promoting hormone insulin-like growth factor-1 (IGF-1) directly regulated placental oGLUT1 and oGLUT3 gene expression.
CHAPTER 2

MATERIALS AND METHODS
2.1 TISSUE COLLECTION

The ontogeny experiment described in Chapter 4 used placental tissue from a study designed by our laboratory to provide maternal, placental and fetal ovine tissues from pregnancies at several gestational ages. The studies described in Chapters 5 and 6 were arranged as collaborations, with tissues from overseas laboratories kindly provided, and those from 'in house' collaborations collected during post-mortem surgery. Professor Jane Harding (Research Centre for Developmental Medicine and Biology, Auckland, NZ) and Professor Bill Hay (University of Colorado Health Sciences Centre, Denver, Colorado, USA) provided placentomes from ovine maternal nutrition and glycemia studies (Chapter 5). Professor Julie Owens (University of Adelaide, Adelaide, Australia), Dr Michael Bauer and Dr Ellen Jensen (Research Centre for Developmental Medicine and Biology, Auckland, NZ) provided tissue from experiments investigating the GH/IGF axes during gestation (Chapter 6). Experimental protocols are described in the relevant chapters, and the chronically-cannulated sheep model employed by studies in Chapters 5 and 6 is outlined below.

2.1.1 Chronically-cannulated sheep model

Surgical and experimental advances made first by Barron’s group in the 1960s and later refined by Meschia, Battaglia and colleagues (Meschia et al., 1980) developed the chronically-cannulated fetal lamb as a useful experimental model for the study of perinatal physiology (Figure 2.1).

![Diagram of the circulation of the pregnant ewe and its conceptus](Image)

**Figure 2.1** Diagram of the circulation of the pregnant ewe and its conceptus (Bell, 1993)

Figure 2.1 illustrates the basic concept of the model. Samples taken from uterine and umbilical arteries and veins allow measurement of the net exchanges of oxygen, nutrients and metabolites by application of the Fick principle (rate of substrate uptake = blood flow x
arteriovenous concentration difference). Nutrient metabolism by fetal and uteroplacental tissues (the conceptus) may be estimated from uterine values, and within the conceptus, umbilical circulation measurements separate the fetus from uteroplacental tissue metabolism. A major advantage of this experimental preparation is that it allows long-term study of conscious, healthy fetuses. It has proved invaluable for investigating a wide range of physiological systems regulating fetal development, including quantitative studies of the nutritional transactions between mother, placenta and fetus. However, the molecular mechanisms underlying such nutrient transport remain unclear, thus providing the raison d'être for this thesis.

2.2 MATERIALS

All radionucleotides were purchased from Amersham, Buckinghamshire, UK. Chemicals used were of analytical/molecular biology grade and basic solutions prepared according to Sambrook et al. (1989) and Titus et al. (1991) (Appendices 1 - 5).

During RNA preparation and analysis, care was taken at all times to avoid introduction of RNases. Glassware was baked at 180°C for 8 h. Solutions were treated with 0.05% diethylpyrocarbonate (DEPC) overnight at room temperature before autoclaving.
2.3 MOLECULAR ANALYSIS

2.3.1 Cloning, subcloning, characterisation and sequence analysis

Methods used for generating ovine GLUT1 and GLUT3 cDNAs are described in section 3.2 of Chapter 3.

2.3.2 Analysis of gene expression

2.3.2.1 Northern analysis

RNA extraction

Tissue was snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using TRIzol™ (Total RNA Isolation Reagent) (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer’s instructions and based on the RNA isolation method developed by Chomczynski and Sacchi (1987). In brief, tissue samples were homogenised in 1 ml of TRIzol™ per 50 – 100 mg tissue using a Polytron (Kinematica, GmbH, Luzern, Switzerland). For ovine placenta, 300 – 400 mg tissue was homogenised in 5 ml TRIzol™. The homogenised samples were incubated at RT for 5 min then 0.2 ml chloroform per 1 ml TRIzol™ was added and mixed vigorously for 15 sec. Samples were incubated at RT for 3 min then centrifuged at 10,000 rpm for 15 min at 4°C. RNA was precipitated from the aqueous layer by addition of 0.5 ml of isopropyl alcohol per 1 ml TRIzol™ used in the initial homogenisation. Samples were incubated at RT for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C. The RNA pellet was washed once with 75% ethanol, air dried and resuspended in (400 μl) DEPC ddH₂O.

The concentration and purity of total RNA was estimated by measuring absorbance (ABS) of UV light at 260 nm and 280 nm using a BeckmanDU®-64 spectrophotometer. An absorbance of 1.0 at 260 nm corresponds to a 40 μg/ml solution of single stranded RNA (Sambrook et al., 1989), and ABS₂₆₀ divided by ABS₂₈₀ provides an estimate of RNA purity and protein contamination. Absorbance readings were taken for duplicate aliquots (10 μl in 1 ml DEPC ddH₂O) of each RNA sample. Samples with ABS₂₆₀/ABS₂₈₀ values of 1.8 or greater were used for Northern analysis.
Random primer labelling of cDNA probes

For Northern analysis a 418 bp PstI-PstI fragment from the 5' end of the oGLUT1 cDNA and the 483 bp oGLUT3 cDNA were labelled with $^{32}$P-dCTP using a Random Primer Labelling Kit (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD, USA). Random primer buffer and 50 ng cDNA template were denatured by boiling for 5 min, quenched on ice, then incubated at 28°C overnight with 2 μl each of 0.5 mM dATP, dGTP, dTTP, 5 μl of 10 mCi/ml $^{32}$P-dCTP (Amersham, Buckinghamshire, UK), and 1.2 μl (5 U) Klenow fragment in a total volume of 50 μl. Unincorporated nucleotides were removed using a Sephadex G-50 spun column (Sambrook et al., 1989). Percent incorporation was calculated by counting β-emission from 2 μl of both pre- and post-column probe in a RackBeta Spectral Liquid Scintillation counter (model LKB 1219). Probes with less than 30% incorporation were discarded.

Northern Protocol

Total RNA (20 μg) was denatured, size separated by gel electrophoresis using a 1% (w/v) agarose gel containing 0.66 M formaldehyde, transferred by capillary action to nylon membrane (Hybond N™, Amersham, Buckinghamshire, UK) overnight and baked at 80°C for 2 h. The integrity and loading of RNA were confirmed by visualisation of Ethidium Bromide stained ribosomal RNA using UV light, and efficiency of transfer of samples was checked by Methylene Blue staining of the membrane (Sambrook et al., 1989; Tijssen, 1993). The Methylene Blue stained 28S rRNA was scanned (Scanmaker IIsp Microtek) and analysed using ImageQuant™ (Molecular Dynamics, Sky Valley, CA, USA). After destaining, membranes were prehybridised in hybridisation buffer (Appendix 2.4) at 42°C for at least 2 h. Ovine cDNAs were labelled with $^{32}$P-dCTP using a DNA Random Priming Labelling Kit, as described, and hybridised to membranes overnight at 42°C. Post hybridisation, membranes were washed once in 2 x SSC for 30 min at RT and twice under high stringency wash conditions (0.1% SDS/0.1 x SSC for 30 min at 60°C). Membranes were then exposed to Kodak X-AR autoradiograph film (Eastman Kodak Co., Rochester, NY, USA) between intensifying screens at -80°C for 3 – 4 d. Relative mRNA abundance was determined by laser scanning densitometry (Personal Densitometer, Molecular Dynamics, Sky Valley, CA, USA) and expressed as a ratio of mRNA:28S rRNA as determined from the Methylene Blue staining. Each Northern analysis was repeated in triplicate.
Statistical analysis

The relative abundance of mRNA levels was determined by densitometric analysis of bands on the resulting autoradiographs (Personal Densitometer, Molecular Dynamics, Sky Valley, CA, USA). To correct for loading, the optical density of mRNA hybridisation signal for each sample was standardised to 28S rRNA (mRNA/28S). The mean control group standardised value was set at 100%, and all other standardised values from the same Northern blot were calculated as percentage values. Combined densitometric quantitation data from replicate blots were presented graphically as mean percentage values ± SEM. Statistical significance was evaluated using unpaired Student’s t-test for comparisons between two means, and One Way ANOVA followed by Bonferroni/Dunn multiple comparison procedure for more than two means. A value of p < 0.05 was interpreted as statistically significant. StatView™ version 4.02 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analysis.

2.3.2.2 In situ hybridisation

In situ hybridisation (ISH) was used to determine the cellular localisation of mRNA. Specific $^{35}$S-labelled probes were hybridised to mRNA on slide mounted tissue sections, and the probe detected using film autoradiography and emulsion autoradiography. Initially, RNA probes were used. However, sense (negative control) probes persisted in giving stronger hybridisation signal than antisense. For this reason oligonucleotide probes were designed and used in all experiments reported here. It was eventually determined that the tissue fixation process was unsuitable for ISH experiments (although worked well for immunohistochemistry), and slide mounted fresh frozen tissue was used in subsequent ISH experiments.

Fresh frozen tissue preparation

Placental tissue was transverse sectioned at collection to include all aspects of placentome morphology. To slow the freezing process and thus preserve cellular architecture, tissue was frozen in an isopentane bath immersed in liquid nitrogen. Frozen sections (10 µm thick) from TissueTek (Miles, Elkhart, IN, USA) embedded tissue were melted on polysine™ (Esco, Biolab Scientific, NZ) glass slides, and stored at -80°C until used for ISH. Prior to hybridisation, sections were fixed in 4% paraformaldehyde/0.1 M PBS for 10 min, washed 3 x 5 min (0.1 M phosphate buffer, Appendix 3.2), dehydrated in 100% ethanol for 5 min, and air dried for 10 min. Slides were vacuum dried for at least 2 h prior to hybridisation.
Oligonucleotide probe preparation and ISH

Specific 48 bp oligonucleotides (oligos) complementary to either 1208 – 1255 bp oGLUT1 (5’-CCCACGCAGCTTCTTCAGCACGCTCTTGCCCGGTTCTCCTCGTTCG- 3’) (Currie et al., 1997), or 3024-3071 bp oGLUT3 (5’-ATGTCCTGAGCCACGTCTCGG-TGCCAGAGTCTGAGGATCTCC- 3’) (Bennett et al., 1995) were ordered from GIBCO BRL Custom Primers (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD, USA). Five pmol of each oligo was labelled at the 3’ end with [α-35S] deoxy-ATP (Amersham, Buckinghamshire, UK) using terminal transferase (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD, USA) and purified using a G-50 sephadex spin column.

The labelled oligos were diluted to approximately 5 x 10^5 cpm/50 µl hybridisation buffer (Appendix 3.1). Hybridisation solution (50 µl) was then added to each slide and incubated overnight in a humidified container at 37°C. After hybridisation, sections were washed once briefly in 1 x SSC/10 mM DTT at RT, twice in 1 x SSC/10 mM DTT for 15 min at 60°C, twice in 0.5 x SSC/10 mM DTT for 15 min at 60°C and once in 0.5 x SSC/10 mM DTT for 15 min at RT. After a brief rinse in water the sections were dehydrated in 100% ethanol (2 x 5 min), air dried and exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 3 – 7 d at 4°C.

Liquid emulsion exposure

After exposure to X-ray film, sections were defatted in the following solutions: 5 min in 95% ethanol, 3 x 5 min absolute ethanol, 10 min xylene, 60 min xylene and 3 x 5 min in absolute ethanol and air dried. In a dark room, slides were dipped in liquid emulsion (LM1, Amersham, Buckinghamshire, UK) heated to 42°C. Once the emulsion dried, slides were stored at 4°C in light-tight containers for 10 – 21 d, depending on strength of signal as judged by film autoradiography, then developed in D19 developer (Eastman Kodak Co., Rochester, NY, USA). The sections were counterstained with Ehrlich’s Haematoxylin/Eosin stain, dehydrated, xylene treated and mounted in Depex (Serva, Heidleberg, Germany). Positive cells contained silver grains and were visualised under the light microscope.
ISH controls

RNase A pre-treatment: to demonstrate that oGLUT probes hybridised to oGLUT mRNA, sections were pre-treated with RNase A (20 μg/ml in 20 mM Tris [pH 7.5], 1 mM EDTA) for 30 min at 37°C, before hybridisation with 35S-labelled oligo probe.

Competitive hybridisation with unlabelled oligo: specificity of the oGLUT1 and oGLUT3 antisense oligo probes was tested on consecutive sections by the addition of approximately 300-fold unlabelled oGLUT1 or oGLUT3 oligonucleotide (10 μg/ml) to 35S-labelled probe prior to hybridisation.

Sequence search: the GCG FASTA sequence comparison program was used to compare the 48 mer oligo oGLUT sequences with those published in GenEMBL. Complementary sequence, other than GLUT1 and GLUT3, was not found.

Five prime labelling of 48 mer oligonucleotides: to check specificity of oGLUT1 and oGLUT3 antisense oligonucleotide probes designed for in situ hybridisation experiments, 10 pmol of oligo was labelled at the 5' end with [γ-32P] deoxy-ATP (Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) and hybridised to 20 μg ovine placental total RNA on a Northern blot. The non-hybridised probe was removed by low stringency washes (2 x SSC for 30 min at RT, 2 x SSC/0.1% SDS for 30 min at 60°C) and membranes exposed to Kodak XAR autoradiograph film (Eastman Kodak Co., Rochester, NY, USA) between intensifying screens at -80°C for a minimum of 3 d.
2.4 PROTEIN ANALYSIS

2.4.1 Antibodies

*Anti-rabbit α1 subunit of Na\(^+\)/K\(^+\)-ATPase (mouse monoclonal IgG<sub>1κ</sub>)*
(Upstate Biotechnology Inc., NY, USA)
This antibody was used as a plasma membrane marker. Na\(^+\)/K\(^+\)-ATPase is an integral membrane protein complex responsible for establishing the electrochemical gradients of Na\(^+\) and K\(^+\) ions across the plasma membranes of mammalian cells. The antibody cross reacts with human, monkey, mouse, rat, dog, lamb, pig and *Xenopus* Na\(^+\)/K\(^+\)-ATPase protein. Horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG was used as the secondary antibody to mouse monoclonal Na\(^+\)/K\(^+\)-ATPase during Western analysis.

*Anti-mouse/rat GLUT polyclonal antibodies*
(Alpha Diagnostic Intl. Inc., San Antonio, TX, USA)
Anti-mouse/rat GLUT1 was raised against 13 aa of the C-Terminus (aa 480 – 492; EEL FHP LGA DSQ V) in rabbits. The peptide sequence used for antibody production is 100% identical to mouse, rat, human, rabbit, bovine, porcine and ovine sequence. To control for specificity, non-immune rabbit serum (NRS) was used at the same concentration as the primary antibody. HRP conjugated goat anti-rabbit IgG was used as the secondary antibody for rabbit anti-mouse/rat GLUT1 primary during Western analysis.

Anti-mouse/rat GLUT3 was raised against 12 aa of the C-terminal (aa 481 – 493; NSM QPV KEP GNA). GLUT3 is not highly conserved across species, and the peptide sequence used to generate this antibody shows 100% homology with mouse and rat sequence only. Corresponding C-terminal ovine GLUT3 sequence is NSI QPT KDT NA, which shows less than 60% homology with rodent sequence. The m/rGLUT3 antibody did not cross react with ovine GLUT3 protein.
2.4.2 oGLUT1 protein levels

2.4.2.1 Western analysis

Protein extraction and preparation

GLUT1 proteins may be stored intracellularly, and only become active once translocated to the plasma membrane (Holman and Cushman, 1996). The intracellular population may lead to over-estimation of active GLUT protein levels. For this reason internal and plasma membrane proteins were separated prior to Western analysis of placental GLUT1 protein levels. The protein extraction and separation technique was based on information published by Bilan et al. (1992), Hundal et al. (1994), Klip et al. (1992), and Tsiani et al. (1995). Figure 2.2 outlines the technique.

All protein extraction and separation steps were performed at 4°C. Approximately 500 mg of placental tissue was finely chopped and placed into a 15 ml centrifuge tube containing 5 ml of freshly made homogenisation buffer (Appendix 4.1). Tissue was homogenised using a Polytron (Kinematica, GmbH, Luzern, Switzerland) set at low power to minimise disruption to the plasma membrane. The homogenate was centrifuged at 1,000 g for 10 min (JA 20.1 rotor, Beckman J2-21M centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA). The pellet (P1, Figure 2.2) was resuspended in fresh homogenisation buffer, homogenised and centrifuged at 1,000 g for 10 min. The pellet (P2) was discarded, and supernatants S1 and S2 pooled and divided into two 5 ml aliquots. One aliquot (S2a) was used to obtain total membranes (P3) by centrifugation at 177,000 g for 1 h (SW 50.1 rotor, Beckman L-70 Ultracentrifuge, Beckman Instruments Inc., Palo Alto, CA, USA). The remaining supernatant (S2b) was centrifuged at 31,000 g for 1 h (JA 20.1 rotor, Beckman J2-21M centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA). The resulting supernatant (S3) was used to collect internal membranes (P6) by centrifugation at 177,000 g for 1 h. To isolate plasma membrane proteins, P4 was gently resuspended in 500 µl of homogenisation buffer and placed on a discontinuous sucrose gradient of 1.4 ml each of 32%, 40%, and 50% (wt/wt) sucrose solution in 20 mM HEPES (pH 7.4). After centrifugation at 210,000 g for 2.5 h (Beckman L-70 Ultracentrifuge) the plasma membrane enriched band isolated above the 32% sucrose layer was collected, added 1:9 to homogenisation buffer and centrifuged at 31,000 g for 1 h.
Figure 2.2 Protein extraction method used to isolate cytosolic, total post-nuclear membrane, plasma membrane, and internal membrane preparations for Western analyses (Bilan et al, 1992; Hundal et al, 1994; Klip et al, 1992; Tsiani et al, 1995).
Membrane protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (Sigma Chemical Co., St. Louis, MO, USA) as per manufacturer’s instructions. Western analysis using a monoclonal antibody to the plasma membrane marker Na\(^{+}/K^{+}\)-ATPase (section 2.4.1) was used to confirm successful enrichment of plasma membrane proteins (Bilan et al., 1992; Tsiani et al., 1995).

**Deglycosylation**

Deglycosylation of GLUT1 was performed with the enzyme N-Glycosidase F (Boehringer Mannheim, GmbH, Biochemica, Mannheim, Germany), which cleaves the N-glycan linkage of glycoproteins between asparagine and the carbohydrate chain. GLUT1 protein (20 µg) was denatured by boiling in 10 µl 1% SDS for 2 min. N-Glycosidase F buffer (90 µl: 20 mM sodium phosphate, 50 mM EDTA, 0.2 mM sodium azide, pH 7.2) and 0.5% (v/v) Nonidet P-40 was added, and the mixture boiled again for 2 min. After cooling to 37°C, 0.8 U N-Glycosidase F was added and the mixture incubated overnight at 37°C. The deglycosylated protein was stored at -20°C until use. Carboxypeptidase Y (CpY), a glycoprotein with 4 N-linked oligo-mannose type glycan chains, was used as a control to demonstrate completion of the deglycosylation reaction.

**Western protocol**

A luminol based chemiluminescent detection system (NuGlo™, Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) was used for Western analysis. Enhanced chemiluminescence (ECL) relies on the oxidation of luminol by the horseradish peroxidase conjugated to the secondary antibody in the presence of chemical enhancers such as phenol. The light produced by this reaction peaks after 5 – 20 min, then decays slowly with a half-life of approximately 60 min, and can be visualised through short exposure to X-ray film.

Total membrane, plasma membrane, and intracellular membrane fractions (1.5 – 5 µg) were diluted in reducing sample buffer (Appendix 4.2). Samples were heated at 95°C for 5 min prior to resolving on polyacrylamide gels against low molecular weight markers (Bio-Rad, Hemmel Hempstead, Hertfordshire, UK) and rGLUT1 positive control (42 – 45 kDa: Alpha Diagnostic Intl., San Antonio, TX, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a Protean II apparatus (Bio-Rad, Hemmel Hempstead, Hertfordshire, UK). Separating gels of 7.5% or 12%, and 4% stacking gels were prepared as described in Appendix 4.3, and
run at 110 V/max. mA for 70 min. Once the dye front was 1 cm from the bottom of the gel the apparatus was dismantled and the gels placed to equilibrate in cold Towbins transfer buffer (Appendix 4.5) for 10 min. The protein was maintained in chilled transfer buffer and transferred to Protran Nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a trans-blot apparatus (Bio-Rad, Hemmel Hempstead, Hertfordshire, UK) at 100 V/max. mA for 1 h.

After transfer, membranes were dried at 37°C for 5 min then air dried for 30 min, and protein visualised using StainAll Dye (Alpha Diagnostics Intl. Inc., San Antonio, TX, USA). Once MW markers were noted and efficiency of transfer determined, membranes were destained (0.01 M PBS) and incubated overnight at 4°C in blocking solution (5% nonfat milkpowder/0.01 M PBS). Next day, after 2 x 5 min washes in wash buffer (0.01 M PBS/0.1% Tween 20), the membranes were incubated in anti-Na⁺/K⁺-ATPase monoclonal antibody (1:2000 dilution) or anti-GLUT1 polyclonal antibody (1:5000 dilution) in antibody diluent buffer (0.01 M PBS/0.05% Tween 20) for 1 h at RT. Unbound primary antibody was removed by 4 x 5 min in wash buffer, then membranes were incubated with horseradish peroxidase-labelled secondary antibody (1:2000 and 1:5000 dilution respectively) for 1 h at RT. Membranes were washed 4 x 5 min in wash buffer and incubated in ECL substrate (Solution A: Solution B 1:1) for 1 min. The reaction was visualised by autoradiography using Kodak XAR film (Eastman Kodak Co., Rochester, NY, USA), and quantified by laser scanning densitometry (Personal Densitometer, Molecular Dynamics, Sky Valley, CA, USA).

Statistical analysis
The mean control group optical density was set at 100%, and all other values from the same Western blot were calculated as percentage values. Combined densitometric quantitation data from replicate blots were presented graphically as mean percentage values ± SEM. Statistical significance was evaluated using One Way ANOVA followed by Bonferroni/Dunn multiple comparison procedure. A value of p < 0.05 was interpreted as statistically significant. StatView™ version 4.02 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analysis.

2.4.2.2 Immunohistochemistry
Immunohistochemistry was performed using the rabbit anti-rat/mouse GLUT1 primary antibody and a Vectastain Elite ABC kit (Avidin and Biotinylated horseradish peroxidase
macromolecular complex, Vector laboratories, Burlingame, CA, USA) which contains a biotinylated goat anti-rabbit secondary antibody, and uses the avidin-biotin horseradish peroxidase detection method. Incubations were performed at RT and 5 μm paraffin embedded sections mounted on 0.01% (w/v) poly-L-lysine (Sigma, St Louis, MO, USA) coated slides were used (Appendix 5.1).

*Paraffin embedded tissue preparation*

Placental tissue was transverse sectioned at collection to include all aspects of placentome morphology and immediately fixed in 4% paraformaldehyde/0.1M PBS (pH 7.4). After 48 h fixation the tissue was embedded in paraffin (Appendix 5.1). Sections (5 μm) were mounted on 0.01% (w/v) poly-L-lysine (P8920, Sigma, St Louis, MO, USA) treated microscope slides and stored at RT.

Embedded sections were deparaffinised in Xylene (2 x 10 min), rehydrated in a descending alcohol series (2 x 2 min) and washed in 0.01 M PBS (5 min). Frozen sections were fixed in 4% paraformaldehyde/0.1 M PBS for 30 min, and then washed 3 x 5 min in PBS/0.2% Triton X-100. Both embedded and frozen tissues were incubated in 1% hydrogen peroxide in 100% methanol for 30 min to block endogenous peroxidase activity. Tissue was washed in 0.01 M PBS (2 x 5 min) and incubated in blocking solution (1.5% normal serum from the species in which the secondary antibody was raised [ie, non-immune goat serum], in 0.01 M PBS) for 1 h at 37°C to block non-specific binding. Sections were incubated with the primary antibody (rabbit anti-rat/mouse GLUT1 at 1:4000 in 0.01 M PBS) overnight at RT in a humidified container. Following incubation, sections were washed in 0.01 M PBS (3 x 5 min) and incubated in secondary antibody (biotinylated goat anti-rabbit serum at 1:100 dilution in blocking solution) at 37°C for 1 h in a humid container. Following a further wash in 0.01 M PBS (3 x 5 min), sections were incubated with avidin-biotin complex (ABC reagent) at 37°C for 1 h. After washing 3 x 5 min in 0.01 M PBS, sections were exposed to the chromogen diamino benzidine (DAB) (Sigma, St Louis, MO, USA) causing the immunoreactive cells to label brown. Sections were rinsed in water, stained using Ehrlich’s haematoxylin, dehydrated in an ascending alcohol series, cleared with xylene and mounted using DPX.

*Controls for immunohistochemistry*

Negative controls for immunohistochemistry were performed by running the above procedure with the primary antibody omitted (0.01 M PBS vehicle only), or with the primary antibody
replaced by the same dilution of non-immune rabbit serum (NRS) (R-9133, Sigma, St Louis, MO, USA). Anti-GLUT1 antibody preincubated with mGLUT1 immunising peptide (0.5 μg/ml; 100 ng mGLUT1 peptide added to 200 μl of 1:4000 mGLUT1 antibody diluted in 0.01 M PBS, and incubated overnight at 4°C) showed no immunoreactivity. Sheep brain, rat placenta and rat brain were used as positive control tissues.
CHAPTER 3

GENERATION OF

OVINE

GLUCOSE TRANSPORTER-1 AND -3 cDNAs
Chapter 3

3.1 INTRODUCTION

The chronically-cannulated fetal sheep provides a useful experimental model for the study of perinatal physiology (Battaglia and Meschia, 1986; Meschia et al., 1980), and is widely used for the study of nutritional transactions between mother, placenta and fetus during development (Harding and Johnston, 1995; Hay, 1999; Owens, 1991). In spite of the progress this model has allowed towards understanding placental nutrient transfer during pregnancy, the underlying molecular transport mechanisms are still relatively unknown. For this reason, the unifying aim of this thesis was to study the molecular regulation of a key placental nutrient transport system; namely the glucose transporter (GLUT) proteins. When this project was started the ovine GLUT cDNAs necessary for the study of GLUT gene expression in sheep did not exist. Therefore, the generation of these ‘molecular tools’ was established as the initial, and pivotal, aim of the thesis.

There are six known functional glucose transporter proteins. They have distinct tissue-specific patterns of expression and were originally characterised by their major site of expression. In placenta, gene expression of two GLUT isoforms, GLUT1 and GLUT3, has been demonstrated in both the human and rat (Bell et al., 1990; Gould and Holman, 1993; Zhou and Bondy, 1993). These two transporters coexist in tissues which exhibit high glucose demand (brain, placenta, tumour tissue), and it has been suggested that they act in tandem to meet the energy demands of such tissues (Gould and Holman, 1993). Ovine GLUT1 and GLUT3 were therefore selected as primary candidates for cloning, although the presence of other hexose transporter proteins in ovine placenta remained a possibility due to the unique nature of ruminant dietary physiology.

This chapter describes the isolation and characterisation of ovine GLUT1 and GLUT3 cDNAs, their nucleotide and deduced amino acid sequences, and homology with other species. Ovine GLUT1 was isolated from a Clontech adult sheep liver λgt10 cDNA library by hybridisation screening using a labelled rat GLUT1 cDNA probe (kindly provided by Dr C Roberts, NIH, Bethesda, USA). An oGLUT3 cDNA was amplified from placental total RNA by reverse transcriptase polymerase chain reaction (RT-PCR). Sequence information (appendix 6) arising from this study has been published (Currie et al., 1997), and submitted to the GenBank™/EMBL Data Bank under accession numbers U89029 (oGLUT1) and U89030 (oGLUT3).
3.2 MATERIALS AND METHODS

3.2.1 oGLUT1

3.2.1.1 cDNA library screen

A 436 bp rat GLUT1 cDNA fragment encoding aa 272 – 708 of the mature rGLUT1 (Dr C Roberts, NIH, Bethesda, USA) was used to screen a λgt10 adult sheep liver cDNA library (Clontech Laboratories, Inc., Palo Alto, CA, USA). Duplicate plaque lifts from the library (40,000 pfu per 150 mm² plate) were baked at 80°C for 2 h, prehybridised with 5 x SSPE, 0.5% SDS, 5 x Denhardts and 100 μg/ml denatured salmon sperm DNA at 42°C for 2 h, then hybridised with 32P-labelled rGLUT1 cDNA overnight at 65°C. Filters were washed at low stringency (2 x SSPE/0.1% SDS at RT twice for 10 min, then 1 x SSPE/0.1% SDS at 65°C for 15 min) and exposed to Kodak XAR autoradiography film (Eastman Kodak Co., Rochester, NY, USA) between intensifying screens for 2 – 3 d at -80°C. Positive plaques from the primary screening were purified to homogeneity by subsequent secondary and tertiary screening using the 32P-labelled rGLUT1 cDNA. The final uniform positive phage was amplified and the λgt10 DNA purified, digested with EcoRI, and electrophoresed on a 1% agarose gel. An ~2.2 kb insert was isolated from the agarose gel using GENECLEAN II® (BIO 101 Inc., La Jolla, CA, USA), subcloned into the EcoRI restriction site of pBluescript®II KS phagemid (Stratagene, La Jolla, CA, USA) and transformed into DH5α cells. Blue-white selection allowed recombinant colonies to be selected for amplification.

3.2.1.2 Subcloning and nucleotide sequence analysis

Purified cDNA fragments resulting from restriction digestion of the ~2.2 kb clone with a combination of EcoRI, PstI, Smal and PvuII were subcloned into pBluescript®II KS at appropriate restriction sites and transformed into DH5α cells. Recombinant plasmid DNA was isolated for double stranded automated sequencing (The Centre for Gene Technology, School of Biological Sciences, University of Auckland, Auckland, NZ) using the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) with M13 forward or reverse sequencing primers.

3.2.1.3 Southern analysis

Amplified DNA from both the λgt10 clone (λGT1) and pBluescript®II KS subclone (pGT1) was purified, digested with EcoRI and electrophoresed on a 0.8% agarose gel. The DNA was
transferred by capillary action overnight to Hybond N™ nylon membrane (Amersham, Buckinghamshire, UK) as per the manufacturer’s instructions. The membrane was prehybridised (5 x SSPE, 0.5% SDS, 5 x Denhardts and 100 µg/ml denatured salmon sperm DNA) at 65°C for 2 h, then hybridised with either 32P-labelled rGLUT1 or rGLUT2 cDNAs overnight at 65°C. Membranes were washed at low stringency (2 x SSPE/0.1% SDS at RT twice for 10 min, then 1 x SSPE/0.1% SDS at 65°C for 15 min) and exposed to Kodak XAR autoradiography film (Eastman Kodak Co., Rochester, NY, USA) between intensifying screens overnight at -80°C.

3.2.2 oGLUT3

3.2.2.1 Primer design
Primer design was based on comparison of published murine (Nagamatsu et al., 1992) and human (Kayano et al., 1988) GLUT3 sequences. Forward (16mer: 5’ - ACTCTTTGTCAACCGC- 3’) and reverse (16mer: 5’ -GCTTCTCCTGTGACAT- 3’) primers corresponded respectively to nucleotides 325 – 340 and 824 – 839 of the mouse GLUT3 cDNA, and the predicted size of the amplified fragment was 483 bp (Christchurch Medical School, Christchurch, NZ).

3.2.2.2 RT-PCR
Total RNA (5 µg) extracted from late gestation (135 d) sheep placenta was reverse transcribed using 400 units Superscript RNaseH Reverse Transcriptase (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD, USA), 0.5 µg oligo (dT) primer (Pharmacia, Bromma, Sweden), 0.5 mM dNTP (Pharmacia, Bromma, Sweden), 50 mM Tris-HCl (pH 8.3), 75 mM KCl and 3 mM MgCl₂ in a total volume of 20 µl. The reactions were incubated for 90 min at 42°C. The resulting cDNA (5 µl) was amplified by 2 U Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in a final volume of 50 µl containing 1 µM of each oligonucleotide primer, 200 µM dNTP, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% Triton X-100. PCR amplification was carried out in an automated thermocycler (Techne PHC-3, Cambridge, UK) with the following thermocycle profile: 3 min at 94°C (denature); 30 cycles of 40 sec at 94°C, 40 sec at 50°C, and 1 min at 72°C; then 10 min at 72°C (final extension).

Amplified PCR products were ligated into the SmaI site of pBluescript®II KS. Transformed colonies were identified by blue-white colour selection, amplified and plasmid DNA isolated.
for double-stranded sequencing by the dideoxynucleotide chain-termination method as described previously for GLUT1 (The Centre for Gene Technology, School of Biological Sciences, University of Auckland, NZ).

3.2.3 Sequence analysis and databank entry

DNA sequence analysis was performed using the Wisconsin Sequence Analysis Package™ (version 9.1, Genetics Computer Group [GCG], Madison, Wisconsin, USA). Sequences were submitted to the GenBank™/EMBL Data Bank via the internet using the BankIt submission form found at http://www.ncbi.nlm.nih.gov.
3.3 RESULTS

3.3.1 oGLUT1 cDNA cloning and sequence determination

Approximately $5 \times 10^5$ recombinants from an adult sheep liver $\lambda$gt10 cDNA library were screened at low stringency with a 436 bp $^{32}$P-labelled rat GLUT1 cDNA. A positive plaque from the primary screen was purified to homogeneity by subsequent secondary and tertiary screening using the rGLUT1 cDNA probe (Figure 3.1). A single clone (AGT1) was isolated and the ~2.2 kb insert released by digestion with EcoRI. Southern analysis demonstrated that $^{32}$P-labelled rGLUT1 hybridised to this insert, and confirmed that the isolate contained an ovine equivalent of GLUT1 (Figure 3.2). Since GLUT2 is the predominant isoform in adult liver from which the cDNA library was made, purified DNA from the recombinant pBluescript®II KS vector containing the ~2.2 kb isolate was subjected to Southern analysis using $^{32}$P-labelled rGLUT1 and rGLUT2 cDNA probes. Rat GLUT1, but not rGLUT2, hybridised to the putative oGLUT1 insert, confirming the clone was the ovine equivalent of GLUT1 (Figure 3.3).

The recombinant pBluescript®II KS plasmid containing the ~2.2 kb clone (pGT1) was restriction mapped, and nine fragments (pGT1-19) were subcloned into pBluescript®II KS after appropriate digestion with a combination of EcoRI, SmaI, PstI and PvuII restriction enzymes (Figure 3.4). Sequencing reactions were performed for both strands of all nine fragments using M13 forward and reverse primers and sequences entered into the fragment assembly system in Wisconsin Sequence Analysis Package™ (version 9.1, GCG, Madison, Wisconsin, USA). This series of related programs was used to assemble overlapping nucleotide sequence fragments into one continuous consensus sequence (Figure 3.5).

A partial sequence of 1600 nucleotides was obtained for the original ~2.2 kb $\lambda$GT1 clone (Figure 3.6). Comparison with a full length (2533 bp) bovine GLUT1 cDNA (Boado and Pardridge, 1990) revealed that the oGLUT1 sequence corresponded to nucleotides 477 – 2079 of bGLUT1, thus lacking approximately 477 nucleotides of 5’ and 454 nucleotides of 3’ sequence. The oGLUT1 sequence contained 1170 nucleotides of open reading frame (390 aa) followed by a stop codon and 426 nucleotides of the 3’ untranslated region (UTR). The 3’ UTR contained a TA rich region of 42 b from nucleotides 1394 – 1435 (Figure 3.6). The 1600 b oGLUT1 sequence showed 97% identity with the corresponding region of bGLUT1 at the nucleotide level. A further 300 b of 3’ cDNA sequence was analysed (Figure 3.5). However,
some physical property of the 3' region of the cDNA (possibly secondary structures) made automated sequencing difficult, and many of these nucleotides were read successfully only once. For this reason the terminal 3' sequence data was not included in the final sequence.

Comparison of deduced amino acid sequences (Figure 3.7) demonstrated that the 390 aa sequence predicted for oGLUT1 corresponded to 103 – 493 aa in bGLUT1 with 99% identity, and demonstrated 97 – 98% identity with corresponding regions of human and rat peptide sequences.

A 417 bp fragment (subclone pGT11, Figure 3.4) corresponding to nucleotides 473 – 889 in the bovine GLUT1 sequence was used as a cDNA probe in subsequent Northern analysis. This region was chosen due to its similarity to previously published rat GLUT1 cDNA fragments used as hybridisation probes (Werner et al., 1989; Zhou and Bondy, 1993).

3.3.2 oGLUT3 RT-PCR cDNA cloning and sequence determination

A 515 bp RT-PCR product, which included oGLUT3 primers and ovine cDNA (Figure 3.8, lane 2), was purified and ligated into the SmaI site of pBluescript®II KS. Sequence analysis revealed a 483 bp ovine cDNA (Figure 3.9) that showed 99% identity to nucleotides 377 – 859 of a full length sheep GLUT3 mRNA sequence published in GenBank™/EMBL Data Bank accession number L39214 (Bennett et al., 1995). The deduced amino acid sequence (161 aa) for the oGLUT3 RT-PCR product (Figure 3.10) corresponded with 100% identity to 88 – 248 aa of the full length sheep deduced GLUT3 amino acid sequence, and demonstrated 88% and 84% identity with corresponding regions of the deduced amino acid sequences of the human (Kayano et al., 1988) and mouse (Nagamatsu et al., 1992) GLUT3 sequences used to design oGLUT3 primers.
Figure 3.1 Nylon membranes showing the isolation and purification of oGLUT1 from a lambda gt10 adult sheep liver cDNA library by screening with a $^{32}$P-labelled rGLUT1 cDNA. A positive plaque (indicated by arrow) identified in the primary screen (A) was purified to homogeneity by subsequent secondary (B) and tertiary (C) rounds of screening and selection.
Figure 3.2 Southern analysis of the putative oGLUT1 cDNA insert (~2.2 kb) isolated from the lambda gt10 cDNA library. A Agarose gel stained with ethidium bromide. B Southern blot hybridised with $^{32}$P-labelled oGLUT1 cDNA: lane 1, MW BMIII; lane 2, MW Low Mass; lanes 3 and 4, uncut lambda gt10 DNA; and lanes 5-7, lambda gt10 DNA digested with EcoRI to release ~2.2 kb cDNA insert.
Figure 3.3 Southern analysis of putative oGLUT1 (~2.2 kb) cDNA cloned into pBluescript®II KS vector. DNA from selected clones was amplified, digested with EcoRI restriction enzyme, electrophoresed on 0.8% agarose gel and transferred to nylon membrane. A Ethidium bromide stained gel showing vector and ~2.2 kb insert. B Nylon membrane hybridised with $^{32}$P-labelled rGLUT2. C Nylon membrane hybridised with $^{32}$P-labelled rGLUT1.
Figure 3.4  A Sequencing strategy for oGLUT1 cDNA. cDNAs subcloned into pBluescript® II KS are designated pGT clones. Arrows indicate sequencing reactions performed with forward or reverse M13 universal primers. B Partial restriction map for oGLUT1 cDNA.
Figure 3.5 Computer generated overview of oGLUT1 sequence assembly project. Dotted arrows (2-15) represent sequence data from 14 cDNA fragments which were aligned using the GCG fragment assembly program to produce a consensus sequence (C) for oGLUT1 cDNA.
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Figure 3.5 Partial nucleotide and deduced amino acid sequences for oGLUT1 cDNA. Amino acids are represented by one letter codes below the codons. The asterisk indicates the stop codon. The TA rich region in the 3' UTR is shown in bold lettering.

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Figure 3.7 Comparison of deduced amino acids sequence of ovine, bovine (Boado and Pardridge, 1990), human (Mueckler et al, 1985) and rat (Birbaum et al, 1986) GLUT1. Amino acids are indicated by their single letter abbreviations. Boxes indicate the eleven positions where variations occur in the amino acid sequences of one or more of the four species.
Figure 3.8 Ethidium bromide stained 2% agarose gel showing a single oGLUT3 product after RT-PCR amplification: lane 1, MW marker; lane 2, 515 bp oGLUT3 product; lane 3, PCR water control; and lane 4, MW marker.
Figure 3.9 Partial nucleotide and deduced amino acid sequences for oGLUT3. Amino acids are represented by one letter codes below the codons.
**Figure 3.10** Comparison of the deduced amino acid sequence of the ovine (1 - 161 aa) RT-PCR product with published sheep (Bennett et al, 1995), human (Kayano et al, 1988) and mouse (Nagamatsu et al, 1992) GLUT3 deduced amino acid sequences (88 - 248 aa). Amino acids are indicated by their single letter abbreviations. Boxes indicate the positions where variations occur in one or more of the four amino acid sequences. Bold lines indicate sequence coding for membrane spanning regions 3, 4, 5 and 6, and the intracellular loop.
Chapter 3

3.4 DISCUSSION

The present study describes partial sequence for ovine GLUT1 and GLUT3 cDNAs. We used classical cloning techniques to isolate an ~2.2 kb oGLUT1 cDNA. Comparison of sequence data obtained from this clone with a full length bovine GLUT1 sequence (Boado and Pardridge, 1990) demonstrated extremely high homology in both nucleotide (97%) and deduced amino acid (99.5%) sequences for a 1600 b sequence corresponding to nucleotides 477 – 2079 in the 2533 bp bGLUT1 sequence. This comparison demonstrated that the oGLUT1 cDNA isolated in our study was not full length, lacking approximately 477 nucleotides at the 5' end. In addition, approximately 454 b of 3' sequence data were lost due to difficulty in obtaining reverse primer sequence from the 3' end of the 3' UTR.

The 426 bp of 3' UTR sequence data obtained for the oGLUT1 cDNA contains a 42 b AT-rich region from nucleotides 1394 – 1435, which is also found in the bovine (Boado and Pardridge, 1990), human (Mueckler et al., 1985), and rat (Birnbaum et al., 1986) 3' UTR of the GLUT1 gene. It is thought that the 3' UTR may play an important role in post-transcriptional regulation by controlling mRNA stability or rate of degradation (McGowan et al., 1995b). Although the precise mechanism(s) involved have yet to be defined, these AU-rich elements may provide sites for specific interactions with cytosolic proteins. These RNA-protein complexes may act to target the mRNA for early degradation, or increase mRNA stability and half-life by protecting the mRNA from degradation. The capacity to control post-transcriptional regulation in combination with the ability to regulate transcriptional activation would allow precise and rapid control of GLUT1 gene regulation, and may protect the fetus from fluctuations in maternal nutrient supply.

The predicted 390 aa sequence obtained for oGLUT1 showed high homology with corresponding regions in bovine, human and rat (97 – 99% identity) deduced amino acid sequences, with the oGLUT1 sequence differing from bGLUT1 at only one amino acid (Q258). In total, the deduced amino acid sequence for oGLUT1 differed at eleven positions from published bovine, human or rat (Birnbaum et al., 1986; Boado and Pardridge, 1990; Mueckler et al., 1985) deduced amino acid sequences. However, none of these changes alter the biochemical characteristics of the protein, confirming the high degree of cross-species conservation observed for the GLUT1 gene (Pessin and Bell, 1992).
The same degree of sequence conservation is not apparent in GLUT3 (Nagamatsu et al., 1992; Pessin and Bell, 1992). Although the RT-PCR product cloned in this study was designed to incorporate a 161 aa fragment of GLUT3 with high cross-species homology, sequence identity with mouse and human GLUT3 deduced amino acid sequences was comparatively low at 84% and 88% respectively. It has been proposed that the amino terminal half of transporter proteins contains the substrate binding site, and that transport occurs by the alternate exposure of this site to the extracellular and intracellular surfaces of the cell membrane due to conformational changes induced by substrate binding (McGowan et al., 1995b). The cross-species differences observed in deduced amino acid sequence between the second membrane spanning region and the intracellular loop may therefore result in species specific differences in protein conformation and/or transport rate.
3.5 SUMMARY AND FUTURE WORK

The objective of this study was to isolate, clone and sequence ovine specific GLUT1 and GLUT3 cDNAs for use in subsequent gene expression studies. The objective was successfully achieved, but with the benefit of experience it is clear that an RT-PCR based approach using degenerate PCR primers may have been easier and quicker. Library screening and analysis of 1600 bp of oGLUT1 cDNA sequence confirmed that oGLUT1 showed high homology with GLUT1 in other mammalian species (97 – 99% identity). This provided valuable data (and experience), but proved time consuming. Ovine GLUT3 was isolated by RT-PCR. This allowed rapid generation of a cDNA suitable for use in expression studies, with sufficient sequence information to confirm that oGLUT3 did not have the same high cross-species homology as GLUT1 (84 – 88% identity). For the purposes of the gene expression studies that were central to this thesis, isolation of small (~500 bp) oGLUT1 and oGLUT3 cDNA sequences would have been sufficient.

An added advantage of RT-PCR based homology screening is that ovine placental RNA could have been simultaneously screened for other GLUT isoforms. GLUT1 and GLUT3 were targeted for study in ovine placenta because they predominate in rodent and human placenta (Hauguel-de Mouzon et al., 1997; Zhou and Bondy, 1993). However, other GLUT isoforms may be present in ovine placenta. Improved detection techniques have recently demonstrated the presence of GLUT2 and GLUT4 in human placenta (Quraishi and Illsley, 1999; Xing et al., 1998), and the unique nature of ruminant dietary physiology (Brockman, 1993) indicates that alternate GLUT isoforms may play important roles in ovine placental hexose transport. For this reason, any future cloning studies should focus on screening ovine placenta for alternative oGLUT isoforms.
CHAPTER 4

oGLUT1 AND oGLUT3

EXPRESSION AND LOCALISATION

DURING PLACENTAL DEVELOPMENT
4.1 INTRODUCTION

In sheep, as in most mammalian species, placental growth occurs predominantly in the first half of gestation (Ehrhardt and Bell, 1995; Schneider, 1996). By 90 d of gestation (term ~145 d) placental growth is largely complete; placental weight and DNA content are maximal, and the placental cotyledons weigh approximately three times as much as the fetus (Battaglia and Meschia, 1986). From mid gestation until term placental weight remains constant and may even decrease slightly (Schneider, 1996). In contrast, the fetus grows rapidly during the second half of gestation. From mid gestation to term the fetal mass increases more than 10-fold (Molina et al., 1991), and at term the fetus weighs more than ten times as much as the placental cotyledons (Bell et al., 1986).

The markedly different growth patterns of fetus and placenta have important implications for the ability of the placenta to maintain an adequate glucose supply to the fetus during the second half of gestation. For the placenta to keep pace with the increasing demands of the growing fetus, and to meet its own considerable glucose requirements (Hay and Wilkening, 1994), placental glucose transport capacity must increase rapidly during the second half of gestation. Part of the increasing glucose requirement is met by an increase in the transplacental glucose concentration gradient driven by altered insulin sensitivities in mother and fetus and by increased uteroplacental glucose consumption (Hay, 1994b). Further increases in placental nutrient transfer are achieved by the placenta undergoing functional maturation during the second half of gestation. Gross morphological and physiological changes have been observed including increased uterine and umbilical blood flow, decreased diffusion distances between maternal and fetal circulations, and expansion of the placental villous surface area (Owens, 1991; Schneider, 1996). However, at the molecular level, the developmental regulation of specific placental nutrient transport systems remains unclear.

Therefore, the objective of studies described in Chapter 4 was to define developmental regulation of oGLUT1 and oGLUT3 during normal ovine placental growth and maturation. Although previous studies had demonstrated a fivefold increase in glucose transport capacity during ovine placental maturation (Molina et al., 1991), it remained unclear whether this was due to villous membrane expansion, or to a concomitant increase in transporter concentration per unit membrane surface area (Hay, 1994b). Furthermore, little was known of the relative
abundance of the individual transporters (oGLUT1 and oGLUT3) or their localisation during ovine placental development.

The initial aims of this study were to measure the relative abundance and identify the cellular location of oGLUT1 and oGLUT3 mRNA in early, mid, and late gestation ovine placenta. As literature emerged demonstrating the importance of GLUT post-transcriptional regulation (McGowan et al., 1995b), these objectives were extended to include measurement of the relative abundance and cellular location of oGLUT1 and oGLUT3 protein in ovine placenta.

The ovine specific cDNAs generated (Chapter 3) were used as $^{32}$P-labelled probes in Northern analyses of oGLUT1 and oGLUT3 mRNA abundance in sheep placenta at d 45, 60, 90, 120, and 138 of gestation. Ovine GLUT1 and GLUT3 specific $^{35}$S-labelled oligonucleotide (48 mer) probes and in situ hybridisation (ISH) were used to determine GLUT1 and GLUT3 transporter mRNA localisation at d 45, 60, 90, 120, and 138 of gestation. Commercially available polyclonal antibodies (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) raised against the C-terminal 12 – 15 amino acids of mouse GLUT1 and GLUT3 were tested for cross-reactivity on sheep placental tissue. Anti-mouse GLUT1 antibody (mGLUT1) was found to cross-react with oGLUT1 protein, and was used for Western analysis and immunohistochemical localisation of oGLUT1 protein in early (d 45) to late (d 138) gestation ovine placenta. Preliminary tests demonstrated anti-mouse GLUT3 antibody (mGLUT3) did not cross-react with oGLUT3 protein, and therefore could not be used to investigate oGLUT3 protein concentration or localisation.
4.2 METHODS

4.2.1 Experimental protocol and tissue collection

Thirty pregnant Coopworth-Border crossbred ewes of known gestational age were kept on farm pasture until the day of tissue collection to minimise stress caused by change in diet and conditions. Six pregnant ewes were killed by overdose of pentobarbitone at each of five gestational ages selected for study (d 45, 60, 90, 120 and 138 of gestation). Table 4.1 lists the number of fetuses from each gestational age.

Table 4.1 Number of singleton and twin pregnancies at each gestational age

<table>
<thead>
<tr>
<th>Gestational age</th>
<th>Singleton pregnancies</th>
<th>Twin pregnancies</th>
<th>Total number of fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 45</td>
<td>2</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>d 60</td>
<td>3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>d 90</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>d 120</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>d 138</td>
<td>5</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

All fetal weight measurements were included in statistical analysis. In twin pregnancies, tissues were collected from one fetus. Fetal weight and total placental weight (defined as combined wet weight of uterus, membranes and placentomes) were recorded, and individual placentomes weighed and counted. Tissue samples from fetal heart, lung, liver, kidney and placentome were snap frozen in liquid nitrogen and stored at -80°C for RNA extraction. Additional samples were collected for immunohistochemistry and ISH and were fixed in 4% paraformaldehyde and embedded in paraffin wax according to the protocol in Appendix 5.1. Maternal and fetal arterial blood and amniotic fluid samples were collected and stored on ice. Within 30 min glucose and lactate concentrations were measured (Yellow Springs Instruments, Yellow Springs, OH, USA), then samples were centrifuged at 6500 rpm for 15 min at 4°C (Beckman J2-21M centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA). Blood plasma and amniotic fluid samples were stored at -20°C. This experiment was approved by Auckland University Animal Ethics Committee.
4.3 RESULTS

4.3.1 Post-mortem animal data

4.3.1.1 Placental and fetal weight

Figure 4.1 presents placental and fetal wet weight measurements taken at d 45, 60, 90, 120 and 138 of gestation. Total placental wet weight increased more than 34-fold from d 45 to d 60 (18 ± 10 to 624 ± 332 g, *p < 0.05), with no further change observed from d 60 to d 138 (term d 145 ± 2). Fetal wet weight increased rapidly from d 45 to d 138 (4 ± 0.5 to 5481 ± 715 g, *p < 0.05, **p < 0.001 where asterisks indicate level of significance).

4.3.1.2 Fetal tissue weights

Fetal heart, liver, lung and kidney wet weight measurements were not significantly different between d 45 and d 60 (Figure 4.2). All fetal tissue wet weights increased rapidly from d 60 to d 138 (p < 0.05).

4.3.1.3 Glucose and lactate concentrations

Figure 4.3 illustrates glucose (A, C and E) and lactate (B, D and F) concentrations in maternal (A, B) and fetal (C, D) blood and amniotic fluid (E, F) sampled at d 45, 90, 120, and 138 of gestation. Fetal blood was not collected at d 45, and glucose/lactate concentrations were not measured at d 60.

Maternal blood glucose concentrations showed no significant change from d 45 (2.7 ± 0.05) to d 120 (3.38 ± 0.18 mM). However, a significant decrease was observed in late gestation at d 138 (Figure 4.3 A, 1.77 ± 0.3 mM, p < 0.05). Amniotic fluid glucose concentrations showed a significant decrease from d 45 to d 120 (1.6 ± 0.19 to 0.35 ± 0.08 mM) and the decrease was maintained at d 138 (Figure 4.3 E, 0.23 ± 0.06 mM, p < 0.05). Fetal blood glucose concentrations were at the lower limits of detection. Mean fetal blood glucose concentration at mid gestation (d 90) was 0.17 mM, and was too low to measure by d 138 (Figure 4.3 C).

No significant changes were observed in either maternal blood or amniotic fluid lactate concentrations (Figure 4.3 B, F). Fetal blood lactate concentrations increased from d 120 (3.75 ± 0.3 mM) to d 138 (5.43 ± 0.27 mM) (Figure 4.3 D, p < 0.05).
4.3.2 oGLUT1 and oGLUT3 mRNA in ovine placenta

4.3.2.1 Northern analysis

Figure 4.4 B presents a representative Northern analysis of ovine placental total RNA, and demonstrates changes observed in the relative abundance of oGLUT1 and oGLUT3 mRNA at d 45, 60, 90, 120, and 138 of gestation. The 417 bp $^{32}$P-labelled oGLUT1 cDNA fragment used for Northern analysis (Figure 3.4 A, pGT11) detected two mRNA transcripts; a major transcript at ~2.8 kb, and a low abundance transcript at ~8 – 10 kb (Figure 4.4 B). The high molecular weight transcript was below the level of detection in early gestation, and the major 2.8 kb transcript was used for all densitometric analyses of oGLUT1. The 483 bp oGLUT3 cDNA probe detected a single mRNA transcript at ~4.0 kb (Figure 4.4 B).

Figure 4.4 A illustrates combined densitometric quantitation data from triplicate Northern analyses of oGLUT1 and oGLUT3 mRNA abundance in ovine placental total RNA. Ovine GLUT1 mRNA was detectable at d 45, and increased in abundance more than 3-fold by d 60. From d 60, oGLUT1 mRNA abundance approximately doubled to reach greatest observed levels at d 120. By d 138 mean oGLUT1 mRNA levels had decreased to about 70% of peak value ($p < 0.05$). Ovine GLUT3 mRNA was detectable by d 45 and continued to increase throughout gestation. Significant increases in mRNA abundance were observed between d 45 – 60 and d 120 – 138 (Figure 4.4 A, $p < 0.05$).

4.3.2.2 In situ hybridisation (ISH)

In situ probe specificity

To check probe specificity, oGLUT1 and oGLUT3 oligonucleotides (48 mer) were 5’end-labelled with [$\gamma^{-32}$P] dATP and hybridised to ovine placental total RNA (20 µg) (Figure 4.5). The oGLUT1 oligonucleotide probe hybridised to an ~2.8 kb transcript and a less abundant higher molecular weight transcript of ~8 – 10 kb. The oGLUT3 oligonucleotide probe hybridised to an ~4.0 kb transcript. Transcript sizes were the same as those observed with [$\alpha^{-32}$P] dCTP labelled oGLUT1 and oGLUT3 cDNA probes (Figure 4.4 B).

To further test probe specificity, $^{35}$S-labelled oGLUT oligonucleotide probes were subjected to competitive hybridisation with unlabelled oGLUT oligonucleotide (Figure 4.6). Consecutive fresh frozen ovine placentome sections (d 126, 10 µm) were hybridised with
hybridisation buffer containing either a mix of unlabelled oGLUT1 or oGLUT3 oligonucleotide and \(^{35}\)S-labelled oligonucleotide at about 300:1 (slides 2 and 3, panels A and B), or with \(^{35}\)S-labelled oligonucleotide probe alone (slide 4, panels A and B). Specific hybridisation of labelled oligonucleotide was blocked by excess of the corresponding unlabelled oligonucleotide (oGLUT1 slide 2, panel A and oGLUT3 slide 3, panel B).

To confirm in situ probes were specific for oGLUT mRNA, target mRNA was degraded by pre-treatment with RNase A (20 \(\mu\)g/ml) prior to hybridisation. Hybridisation of both oGLUT1 and oGLUT3 \(^{35}\)S-labelled oligonucleotide antisense probes was prevented except for a narrow band of intense signal at the margin (Figure 4.6, slide 1, panels A and B).

**Localisation of oGLUT1 and oGLUT3 mRNA in ovine placenta by ISH**

Figures 4.7 to 4.10 show representative ISH experiments on fresh frozen d 126 ovine placenta. Figures 4.7 and 4.8 present photomicrographs of slides hybridised with \(^{35}\)S-labelled oGLUT1 oligonucleotide probe. Figure 4.9 and 4.10 present results from \(^{35}\)S-labelled oGLUT3 oligonucleotide probe. Paired photomicrographs were taken using light field (left) and darkfield (right) illumination of the same area of tissue.

Figure 4.7 depicts a representative oGLUT1 ISH experiment. RNase A pre-treatment (A, B) and competitive hybridisation by excess unlabelled oGLUT1 oligonucleotide (C, D) generated low background hybridisation signal. Hybridisation signal was not blocked by excess unlabelled oGLUT3 oligonucleotide (E, F), demonstrating the specificity of oGLUT1 probe hybridisation. Labelled oGLUT1 probe alone (G, H) gave a strong hybridisation signal which, though present in both maternal and fetal tissue, was localised most strongly to the maternal-fetal barrier. Figure 4.8 A – D presents photomicrographs from Figure 4.7 H at higher magnification (10 x 40). Ovine GLUT1 hybridisation signal appeared strongest over clusters of multiple nuclei at the maternal-fetal interface (Figure 4.8 A and B).

The lower panels in Figure 4.8 (E, F) show low magnification (10 x 10) photomicrographs of oGLUT1 ISH signal from the region where maternal villi emerge from the endometrium. Signal in this region was exceptionally strong and was not blocked by either RNase A pre-treatment or addition of excess cold oligonucleotide (Figure 4.6). Although this failure of control experiments indicates non-specific hybridisation, signal in this region was consistently confined to maternal endometrial tissue and maternal villi. Within the villi it was most strongly
localised to a narrow margin on the maternal-facing side of the maternal-fetal barrier (Figure 4.8 F).

Figure 4.9 depicts a representative oGLUT3 ISH experiment. RNase A pre-treatment (A, B) and competitive hybridisation by excess unlabelled oGLUT3 oligonucleotide (E, F) generated low background hybridisation signal. Hybridisation signal was not blocked by excess unlabelled oGLUT1 oligonucleotide (C, D), demonstrating the specificity of oGLUT3 probe hybridisation. ³⁵S-labelled oGLUT3 probe alone (G, H) gave a strong hybridisation signal which was localised to the maternal-fetal barrier. Figure 4.10 A – D presents photomicrographs from Figure 4.9 D and H at higher magnification (10 x 40). Ovine GLUT3 hybridisation signal appeared most strongly located to the fetal side of the multi-nucleate cell layer (Figure 4.10 D).

ISH was used to investigate localisation of oGLUT1 and oGLUT3 mRNA in ovine placenta during gestational development. Representative photomicrographs demonstrate oGLUT1 (Figure 4.11) and oGLUT3 (Figure 4.12) hybridisation signal on fresh frozen ovine placental tissue sections (10 μm) from d 45 (A, B), d 60 (C, D), d 90 (E, F) and d 138 (G, H) of gestation. In early gestation (d 45), oGLUT1 hybridisation signal was relatively low in both maternal and fetal tissue. Signal was most strongly localised above multi-nucleate cells at the maternal-fetal barrier (Figure 4.11 B). From d 60 to mid gestation (d 90) oGLUT1 hybridisation signal increased in maternal villous tissue, and was most strongly localised to the maternal facing side of the maternal-fetal barrier (Figure 4.11 D, F). In contrast, oGLUT3 hybridisation signal was more restricted, and was most strongly localised to fetal trophoblast cells (Figure 4.12 B, D, and F).

4.3.3 Ovine placental GLUT1 protein

4.3.3.1 Western analysis

mGLUT1 and mGLUT3 antibody cross-reactivity with ovine GLUT proteins

Anti-mouse GLUT1 (mGLUT1) antibody was raised against mouse amino acid sequence with 100% identity to both rat and ovine sequence (section 2.4.1). Consequently the mGLUT1 antibody cross-reacted with ovine GLUT1 protein and was used for both Western analysis and immunohistochemistry. In contrast, the mouse peptide sequence used to raise mGLUT3 antibody showed less than 60% identity with the corresponding ovine sequence, and dot blot
and Western analysis demonstrated that mGLUT3 polyclonal antibody did not cross-react with oGLUT3 (Figure 4.13 A, B).

**Glycosylated versus deglycosylated ovine placental proteins**

Anti-mouse GLUT1 antibody bound to both rat (Figure 4.14 B, lanes 4 – 7) and ovine (lanes 8 – 13) placental total protein with similar efficacy and at similar molecular weights. Furthermore, mGLUT1 antibody bound equally well to glycosylated (rat, lanes 4 – 5; sheep, lanes 8, 9, 10) and deglycosylated (rat, lanes 6 – 7; sheep, lanes 11, 12, 13) protein (Figure 4.14 B). The only apparent difference between species was the molecular weight of two minor bands visible in both species and in the mouse positive control peptide. The higher molecular weight band had a molecular weight of ~80 kDa in ovine and ~75 kDa in rat placental total protein. The lower molecular weight band had a molecular weight of ~50 kDa in ovine and ~55 kDa in rat total placental protein (Figure 4.14 B).

Glycosylation may target proteins to specific cellular destinations (Darnell et al., 1990). Western analysis was used to investigate glycosylation of oGLUT1 proteins isolated from internal and plasma membrane fractions of late gestation (d 138) ovine placenta (Figure 4.14 C). A 12% SDS-PAGE gel was loaded with 5 µg glycosylated (n = 2) and deglycosylated (n = 2) total ovine placental protein samples (lanes 4 – 7), internal membrane protein samples (lanes 8 – 11), and plasma membrane protein samples (lanes 12 – 15). No differences in molecular weights were observed, suggesting that differences in glycosylation, if present, were not large enough to be detected using this method.

**Separation of internal and plasma membrane proteins**

A mouse monoclonal antibody raised against the α subunit of plasma membrane associated Na⁺/K⁺ ATPase protein was used to indicate successful separation of internal and plasma membrane proteins by the method described in section 2.4.2.1. The dotblot in Figure 4.15 demonstrated that mouse Na⁺/K⁺ ATPase monoclonal antibody cross-reacted with native (glycosylated) ovine placental protein, but did not recognise deglycosylated ovine protein. For this reason all subsequent Western analyses comparing total, internal and plasma membrane fractions were carried out using glycosylated (native) ovine placental protein. This allowed each Western analysis to be immunoblotted sequentially with oGLUT1 polyclonal antibody (1:5000, 1:5000), non-immune rabbit serum (NRS) control at the same dilutions (1:5000,
1:5000), and mouse Na⁺/K⁺ ATPase monoclonal antibody (1:2000, 1:2000) (See for example Figure 4.17).

*Western analysis of oGLUT1 levels in ovine placenta*

Figure 4.16 presents data from Western analyses of oGLUT1 protein levels in total post-nuclear protein preparations from ovine placenta at d 45, 90, and 138 gestation. Total placental oGLUT1 protein levels increased throughout gestation, with significant increases observed between d 45 and d 90, and d 90 and d 138 (term d 145 ± 2) (p < 0.0001).

To separate intracellular oGLUT1 protein from activated oGLUT1 protein in the plasma membrane, samples of plasma membrane (PM), internal light microsomal membrane (IM), and total post-nuclear membrane (TM) protein fractions were analysed separately by Western immunoblot for GLUT1 immunoreactivity at d 45, 90 and 138 (Figure 4.17, i – iii, representative Western blots). Figure 4.18 presents densitometric quantitation data from four replicate Western analyses at early (d 45), mid (d 90) and late (d 138) gestation. Relative expression of oGLUT1 immunoreactivity was greater in PM fractions than IM fractions at all age groups studied, and greater than both TM and IM fraction at d 90 and d 138 (Figure 4.18, p=0.0052, p<0.0001, p=0.001). Relative abundance of oGLUT1 protein in the IM fraction remained constant throughout gestation (70 – 88% compared to TM fraction). In contrast, the relative abundance of oGLUT1 protein in the PM fraction increased in mid to late gestation compared to oGLUT1 levels in the TM fraction, with a 73% increase at d 90 and over 100% increase by d 138 (Figure 4.18).

**4.3.3.2 Immunohistochemistry**

*mGLUT1 and mGLUT3 antibody cross-reactivity with ovine and rat tissues*

Ovine placental GLUT immunohistochemistry was carried out using mGLUT polyclonal antibodies (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) and immunoperoxidase based Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) on paraformaldehyde fixed, paraffin embedded ovine placental tissue sections (5 µm) (section 2.4.2.2).

Preliminary tests using primary antibody dilutions from 1:50 to 1:5000 demonstrated that mGLUT3 polyclonal antibody did not cross-react with ovine tissue (Figure 4.19 A), and therefore could not be used to investigate oGLUT3 localisation. Mouse GLUT1 polyclonal
antibody did cross-react with oGLUT1 protein, and the photomicrographs in Figure 4.19 B – D demonstrate the specificity of mGLUT1 immunoreactive signal. NRS used at the same dilution as the mGLUT1 primary antibody (1:4000) gave no signal, demonstrating that the rabbit anti-mouse antibody was specific to GLUT1 (Figure 4.19 B). Pre-absorption of mGLUT antibody (1:4000) with mGLUT1 peptide (0.5 µg/ml) blocked all immuno-signal, further demonstrating the specificity of the mGLUT1 antibody for oGLUT1 protein (Figure 4.19 C). The photomicrograph in Figure 4.19 D shows late gestation (d 138) ovine placental tissue incubated with mGLUT1 primary antibody (1:4000), and demonstrates oGLUT1 immunoreactivity localised to the maternal-fetal border.

Figure 4.20 shows relative expression of immunoreactive GLUT1 protein in a range of tissues known to be positive for GLUT1 protein, including postnatal d 33 Wistar Koyoto rat brain (A, B), gestational d 20 Wistar Koyoto rat placenta (C, D), and late gestation (d 133) fetal sheep brain (E, F). Photomicrographs on the left show tissue incubated with NRS control (1:4000), and on the right consecutive tissue slices incubated with mGLUT1 primary antibody (1:4000). In rat brain, GLUT1 protein was present in vascular endothelium, and was concentrated in the CA1 region of the hippocampus (Figure 4.20 B). In sheep brain, GLUT1 positive staining was strong in vascular endothelium. However, no regional concentrations were seen (Figure 4.20 F). In late gestation rat placenta, GLUT1 immunoreactivity was strongest in the labyrinthine zone, although abundant immuno-signal was also present in the junctional zone and surrounding peripheral giant cells (Figure 4.20 D).

Localisation of oGLUT1 protein in ovine placenta by immunohistochemistry

Immunohistochemistry was used to investigate localisation of oGLUT1 protein in ovine placenta during gestational development. Figure 4.21 presents a series of photomicrographs illustrating oGLUT1 immunoreactivity in ovine placental tissue at d 45 (A, B), d 60 (C, D), d 90 (E, F), d 120 (G, H), and d 138 (I, J), with representative d 120 (K) and d 138 (L) NRS controls. Photomicrographs on the left are low magnification (10 x 10), and those on the right show the same area at higher magnification (10 x 40).

At d 45 of gestation GLUT1 immunoreactivity was restricted to the maternal-fetal barrier, with intense staining surrounding multinucleate cells and cuboidal cells of the invading fetal trophoblast (B). At this early stage of gestation oGLUT1 immunoreactivity was extremely weak or absent in maternal endometrium and maternal villi (A, B). At d 60 GLUT1
immunoreactivity was still relatively low. However, oGLUT1 immuno-signal was observed surrounding multi-nucleate trophoblast cells (D) and was increasingly abundant in maternal endometrium and maternal villi (C, D). By d 90 relative expression of immunoreactive GLUT1 was high, with intense GLUT1 immunoreactivity observed in maternal villi, and in maternal endometrial tissue surrounding the trophoblast cells at the termini of invading fetal villi. In contrast, the intense signal in terminal fetal villi observed at d 45 was no longer apparent (E). GLUT1 immunoreactivity was also present at the syncytial layer forming a barrier between maternal and fetal tissues (E, F). By d 120 a narrow band of intense GLUT1 immunoreactivity was observed adjacent to maternal endometrium. This signal was not seen in placental tissue incubated with NRS control (K). By d 120 maternal and fetal villi were closely interwoven, with GLUT1 immunoreactivity delineating the margins of villi (G). Of the gestational ages studied, binucleate cells were most abundant at d 120, and these large cells were surrounded by GLUT1 immunoreactive signal (H). The narrow band of strong GLUT1 immunoreactivity adjacent to the endometrium was also present at d 138 (I), but was not present in tissue incubated with NRS (L) indicating specific oGLUT1 immunoreactivity.
Figure 4.1 Total placental (A) and fetal (B) wet weight at d 45 (n = 10), d 60 (n = 9), d 90 (n = 6), d 120 (n = 6) and d 138 (n = 7) of ovine gestation. Data is expressed as mean ± SEM. Different letters denote significant differences, and levels of significance are indicated by asterisks (*p < 0.05, ***p < 0.001, ANOVA/Bonferroni Dunn).
Figure 4.2 Post-mortem wet weight data from ovine fetal heart (A), liver (B), lung (C), and kidney (D) at d 45, 60, 90, 120 and 138 of gestation. Data is expressed as mean ± SEM. Different letters denote significant differences (p < 0.05, ANOVA/Bonferroni Dunn).
Figure 4.3 Glucose and lactate concentrations (mmol/l) in maternal (A, B) and fetal (C, D) blood and amniotic fluid (E, F) at d 45, 90, 120 and 138 of ovine pregnancy (n = 6 at each gestational age). Data is expressed as mean ± SEM. Fetal blood was not collected at d 45 and no measurements were taken at d 60. Day 138 fetal glucose concentration was measured at 0 mmol/l. Different letters denote significant differences (p < 0.05, ANOVA/Bonferroni Dunn).
Figure 4.4 Northern analysis of oGLUT1 and oGLUT3 gene expression during placental development. A Densitometric quantitation analysis of oGLUT1 and oGLUT3 mRNA during placental development. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean d 138 value. Graphs present mean percentage values ± SEM from triplicate Northern analyses. Different letters denote significant differences (p < 0.0001, ANOVA/Bonferroni Dunn). B Representative Northern blot demonstrating placental oGLUT1 (8 - 10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA at d 45, 60, 90, 120 and 138 gestation (n = 6 per age group, 20 µg total RNA loaded per sample).
Figure 4.5 Northern analysis of *in situ* probe specificity. Oligonucleotides (48 mer) were 5' end-labelled with $[\gamma^{-32}P]$ dATP and hybridised to 138 ovine placental total RNA (20 μg per sample). A $[\gamma^{-32}P]$-labelled oGLUT1 oligonucleotides hybridised to ~2.8 kb mRNA transcripts. B $[\gamma^{-32}P]$-labelled oGLUT3 oligonucleotides hybridised to ~4.0 kb mRNA transcripts.
Figure 4.6 Autoradiograph of representative *in situ* hybridisation demonstrating probe specificity. A $^{35}$S-labelled oGLUT1 48 mer oligonucleotide and B $^{35}$S-labelled oGLUT3 48 mer oligonucleotide *in situ* hybridisation performed on fresh frozen ovine placenta (d 126). Serial tissue sections (10 μm) on consecutive slides were treated as follows: slides 1, RNase A (20 μg/ml) pre-treatment of tissue prior to $^{35}$S-labelled oligonucleotide probe hybridisation; slides 2, Tissue hybridised to a mix of unlabelled oGLUT1 oligonucleotide (~300-fold excess) and $^{35}$S-labelled oligonucleotide probe; slides 3, Tissue hybridised to a mix of unlabelled oGLUT3 oligonucleotide (~300-fold excess) and $^{35}$S-labelled oligonucleotide probe; slides 4, Tissue hybridised to $^{35}$S-labelled oligonucleotide probe alone.
Figure 4.7 Localisation of oGLUT1 mRNA in fresh frozen sections (10 μm) of ovine placenta (d 126) visualised using 35S-labelled oGLUT1 oligonucleotide (48 mer) probe and in situ hybridisation. Paired photomicrographs of each tissue section were taken using brightfield (left) and darkfield (right) light microscope illumination (magnification 10 x 10). A, B RNaseA pre-treatment prior to oGLUT1 probe hybridisation. C, D Excess (300-fold) unlabelled oGLUT1 oligonucleotide with 35S-labelled oGLUT1 probe. E, F Excess (300-fold) unlabelled oGLUT3 oligonucleotide with 35S-labelled oGLUT1 probe. G, H 35S-labelled oGLUT1 probe alone. Arrows indicate trophoblast cell layer separating maternal (m) and fetal (f) tissue (G).
Figure 4.8 Localisation of oGLUT1 mRNA in fresh frozen sections (10 μm) of ovine placenta (d 126) visualised using 35S-labelled oGLUT1 oligonucleotide (48 mer) probe and in situ hybridisation. Paired photomicrographs of each tissue section were taken using brightfield (left) and darkfield (right) light microscope illumination. The upper four photomicrographs (A - D) present images from Figure 4.7 H at higher magnification (10 x 40). Arrows indicate multi-nucleate cells separating maternal (m) and fetal (f) tissue (A). The lower panels (E, F) illustrate abundant signal observed in maternal endometrium (e) and associated maternal villi (magnification 10 x 10).
Figure 4.9 Localisation of oGLUT3 mRNA in fresh frozen sections (10 μm) of ovine placenta (d 126) visualised using 35S-labelled oGLUT3 oligonucleotide (48 mer) probe and in situ hybridisation. Paired photomicrographs of each tissue section were taken using brightfield (left) and darkfield (right) light microscope illumination (magnification 10 x 10). A, B RNaseA pre-treatment prior to oGLUT3 probe hybridisation. C, D Excess (300-fold) unlabelled oGLUT1 oligonucleotide with 35S-labelled oGLUT3 probe. E, F Excess (300-fold) unlabelled oGLUT3 oligonucleotide with 35S-labelled oGLUT3 probe. G, H 35S-labelled oGLUT3 probe alone. Arrow indicates trophoblast cell layer separating maternal (m) and fetal (f) tissue.
Figure 4.10 Localisation of oGLUT3 mRNA in fresh frozen sections (10 µm) of ovine placenta (d 126) visualised using 35S-labelled oGLUT3 oligonucleotide (48 mer) probe and in situ hybridisation. Paired photomicrographs present brightfield (left) and darkfield (right) images from Figure 4.9 D and H at higher magnification (10 x 40). Arrows indicate intense oGLUT3 hybridisation signal on the fetal-facing side of the syncytiotrophoblast layer (f = fetal tissue, m = maternal tissue).
Figure 4.11 Gestational development of oGLUT1 mRNA localisation in ovine placenta visualised using $^{35}$S-labelled oGLUT1 oligonucleotide (48 mer) probe and in situ hybridisation. Sections (10 μm) were cut from tissue snap frozen in liquid nitrogen. Paired photomicrographs were taken using brightfield (left) and darkfield (right) light microscope illumination. A, B d 45 gestation (10 x 10). C, D d 60 gestation (10 x 20). E, F d 90 gestation (10 x 10). G, H d 138 gestation (10 x 20). Arrows indicate the maternal-fetal barrier.
Figure 4.12 Gestational development of oGLUT3 mRNA localisation in ovine placenta visualised using $^{35}$S-labelled oGLUT3 oligonucleotide (48 mer) probe and in situ hybridisation. Sections (10 μm) were cut from tissue snap frozen in liquid nitrogen. Paired photomicrographs were taken using brightfield (left) and darkfield (right) light microscope illumination. A, B d 45 gestation (10 x 10). C, D d 60 gestation (10 x 10). E, F d 90 gestation (10 x 20). G, H d 138 gestation (10 x 10). Arrows indicate the maternal-fetal barrier.
Figure 4.13 Immunoblot analysis of mGLUT3 polyclonal antibody cross-reactivity with ovine placental total protein. A Immuno dot blot of 4 μg and 10 μg aliquots of ovine total placental protein in reducing sample buffer incubated with primary mGLUT3 antibody dilutions of 1:100 (data not shown), 1:500, 1:1000 or 1:1500, and secondary antibody (goat anti-rabbit) dilutions of 1:2500 or 1:5000. Mouse GLUT3 positive peptide control (12.5 μl) was incubated at 1:1500, 1:5000. B Western blot (7.5% SDS-PAGE) of ovine placental total protein with (i) mGLUT3 polyclonal antibody (1:500, 1:5000), and (ii) NRS control (1:500, 1:5000). MWs (lane 1) are marked to the left of the figure. Lane 2 contained mGLUT1 positive peptide (12.5 μl), lane 3 mGLUT3 positive peptide (12.5 μl). Aliquots (4 μg) of total post-nuclear protein from d 45 (lanes 4 - 7), d 90 (lanes 8 - 11), and d 138 (lanes 12 - 15) ovine placetomes were loaded. Membrane (i) was subsequently stripped and immunoblotted with mGLUT1 polyclonal antibody (1:5000, 1:5000). A 42 - 45 kDa band was observed in mGLUT1 positive peptide (lane 2) and all ovine samples (lanes 4 - 15) (data not shown).
**Figure 4.14** Western analysis of oGLUT1 glycosylation. **A** Control deglycosylation reaction. Coomassie stained Western gel (7.5% SDS-PAGE) with 2.5 μg per lane of the glycoprotein Carboxypeptidase Y (CpY) before (lanes 2, 3) and after (lanes 4, 5) deglycosylation with N-Glycosidase F. **B** Western blot (7.5% SDS-PAGE) analysis of non-treated (lanes 4, 5) and deglycosylated (lanes 6, 7) late gestation rat placenta protein, and non-treated (lanes 8 - 10) and deglycosylated (lanes 11 - 13) d 138 gestation ovine placental protein. Three μg aliquots of total protein were loaded in each lane: lane 1, MW; lane 2, mGLUT1 positive peptide control (12.5 μl); and lane 3, mGLUT3 positive peptide control (12.5 μl). Antibody dilutions were primary mGLUT1 1:5000, goat anti-rabbit secondary antibody 1:5000. A duplicate Western incubated with NRS (1:5000, 1:5000) showed no immunoreactivity (data not shown). **C** Western blot (12% SDS-PAGE) analysis of glycosylated (n = 2) and deglycosylated (n = 2) protein from total (lanes 4 - 7), internal (lanes 8 - 11), and plasma (lanes 12 - 15) membrane fractions isolated from two d 138 ovine placentomes. Five μg of protein was loaded per sample: lane 1, MW; and lane 2, mGLUT1 positive peptide control (12.5 μl).
**Figure 4.15** Immuno dot blot of 4 µg aliquots of deglycosylated (top blot on each membrane strip), and 4 µg and 10 µg aliquots of glycosylated ovine total placental protein incubated with primary mouse monoclonal antibody raised against the alpha 1-subunit of rabbit Na⁺/K⁺ ATPase at dilutions of 1:1000, 1:1500 and 1:2000. Secondary antibody (sheep anti-mouse) dilutions were 1:1000 or 1:2000.
Figure 4.16 Western analysis showing relative expression of immunoreactive GLUT1 in total protein isolated from d 45 (n = 4), d 90 (n = 4), and d 138 (n = 4) ovine placentomes. Aliquots (10 μg) of deglycosylated total membrane protein were electrophoresed on large 7.5% SDS-PAGE gels and transferred to membrane for immunoblotting. A Densitometric quantitation analysis of placental oGLUT1 protein in d 45, d 90, and d 138 ovine placentomes. GLUT1 band optical density was calculated as percentage of mean d 138 value. Graph presents mean percentage values ± SEM from duplicate Western blots. Different letters denote significant differences (p < 0.0001, ANOVA/Bonferroni Dunn). B Representative Western analysis incubated with (i) mGLUT1 polyclonal antibody (1:5000, 1:5000), and (ii) NRS (1:5000, 1:5000) to control for non-specific binding. Lane 1, 20 μl of control mGLUT1 positive peptide.
Figure 4.17 Representative Western analyses showing relative expression of immunoreactive GLUT1 in total (TM, n = 4), internal (IM, n = 4) and plasma (PM, n = 4) membrane fractions isolated from (i) d 45, (ii) d 90, and (iii - v) d 138 ovine placentomes. Aliquots (5 μg) of glycosylated protein were electrophoresed on 7.5% SDS-PAGE gels and transferred to membrane for immunoblotting. Western blots were incubated with (i - iii) mGLUT1 polyclonal antibody (1:5000, 1:5000), (iv) NRS (1:5000, 1:5000) to control for non-specific binding, and (v) a monoclonal antibody raised against plasma membrane specific Na⁺/K⁺ ATPase (1:2000, 1:2000) to demonstrate successful enrichment of the PM protein fraction.
Figure 4.18 Densitometric quantitation analysis of placental oGLUT1 protein in total (TM), internal (IM) and plasma (PM) membrane fractions isolated from d 45 (A), d 90 (B), and d 138 (C) ovine placentomes. GLUT1 autoradiograph band optical density was calculated as percentage of mean total membrane (TM) value. Graph presents mean percentage values ± SEM from four replicate Western blots at each gestational age (see Figure 4.17 i - iii for representative Western blots). Different letters denote significant differences (\( ^{a}p = 0.0052, \ ^{b}p < 0.0001, \ ^{c}p = 0.001 \), ANOVA/Bonferroni Dunn).
Figure 4.19 Immunohistochemical characterisation of mGLUT1 and mGLUT3 polyclonal antibody cross-reactivity with ovine tissue. Fixed and embedded sections (5 μm) of d 138 ovine placentome tissue were immunostained with: A mGLUT3 antibody (1:1000); B Non-immune rabbit serum (NRS, 1:4000); C mGLUT1 antibody (1:4000) preincubated with the immunising mGLUT1 peptide (0.5 μg/ml) (magnification 10 x 10); and D mGLUT1 antibody (1:4000) (magnification 10 x 20).
Figure 4.20 Immunohistochemical localisation of GLUT1 protein. A, B Postnatal d 33 rat brain. C, D d 20 rat placenta. E, F d 133 ovine brain. Fixed and embedded sections (5 μm) were immunostained with: A, C, E NRS (1:4000); B, D, F mGLUT1 polyclonal antibody (1:4000). GLUT1 immunoreactivity was seen: B in vascular endothelium in the CA1 region of the rat hippocampus (arrow); D in the rat placenta in labyrinthine (l) and junctional (j) zones, and surrounding peripheral giant cells (g); and F surrounding blood vessels in ovine brain tissue (arrow) (magnification 10 x 10).
Figure 4.21 (Continued on following page). Immunohistochemical localisation of oGLUT1 protein in ovine placenta at d 45 (A, B), d 60 (C, D), d 90 (E, F), d 120 (G, H), and d 138 (I, J) of gestation. Fixed and embedded sections (5 μm) were immunostained with mGLUT1 polyclonal antibody (1:4000). Consecutive sections were incubated with NRS (1:4000). Photomicrographs K and L present representative NRS control sections for d 120 and d 138 placenta. Hematoxylin stain was used to identify cell nuclei. Eosin counterstain was not used because it reduced the clarity of the brown immunosignal. Labels are m = maternal, f = fetal, e = endometrium. Arrows indicate multi-nucleate trophoblast cells (magnification A, C, E, G, I, K, L 10 x 10; B, D, F, H, J 10 x 40). Photomicrographs are continued on the following page.
Figure 4.21 continued See previous page for legend.
4.4 DISCUSSION

The aim of these studies was to define developmental regulation of oGLUT1 and oGLUT3 during normal ovine placental growth and maturation. Post-mortem measurements confirmed that fetal body weight, fetal organ weight and total placental weight from animals used in the current study fell within previously published parameters for normal fetal and placental development (Ehrhardt and Bell, 1995; Schneider, 1996). Expression and localisation studies revealed that oGLUT1 and oGLUT3 have distinct ontogenic patterns of mRNA abundance and localisation, indicating that the isoforms may play different roles in placental glucose transport. Furthermore, oGLUT1 protein and mRNA levels differed in late gestation, indicating post-transcriptional regulation of oGLUT1 protein expression.

4.4.1 Post-mortem data

Ehrhardt and Bell (1995) have demonstrated that in sheep both wet and dry placental weight (defined as total mass of placentomes) increase rapidly from d 40 to d 75 - 80, and that placental wet weight decreases following this peak. In contrast, maximal placental weight in the current study was achieved by d 60, after which no further significant weight change was observed. In this study, placental weight was defined as the combined wet weight of uterus, membranes and placentomes; a measurement that provided only a rough estimate of placental weight. In addition, small sample size and undetected twin pregnancies may have contributed to the large variation seen in total placental weight, and obscured changes in late gestation placental weight.

In ruminants, volatile acids are the primary products of digestion, and glucose is almost entirely produced by gluconeogenesis (Brockman, 1993). As a consequence normal blood glucose concentrations in adult sheep lie between 1 – 4 mM, and are relatively low compared to human levels of 3.9 – 5.6 mM (Bell, 1993). In this study, mean maternal blood glucose concentrations ranged between 2.7 – 3.4 mM, and showed no change at d 45, 90 and 120. The decreased maternal blood glucose and amniotic fluid glucose concentrations measured at d 138 may reflect increased glucose utilisation for placental metabolism and fetal growth in late gestation (Battaglia and Meschia, 1986).

Fetal blood glucose concentrations were considerably lower than maternal and amniotic fluid levels, with no fetal blood glucose measurements recorded above 0.3 mM. During late
gestation, high glucose consumption by the uteroplacenta and fetus lead to low plasma glucose concentrations in the fetus. This relative fetal hypoglycemia plays an important role in maintaining transplacental glucose flux. However, ovine fetal plasma glucose concentrations in mid- to late-gestation chronically catheterised preparations are usually higher than those observed in the current study, with published concentrations ranging from about 0.8 mM (Harding et al., 1994; Jensen et al., 1999) to 1.1 mM (Das et al., 1999; Hay, 1995b). In the current study, it is unlikely that low fetal blood glucose concentrations were due to disrupted maternal feed intake (maternal glucose supply) because maternal blood glucose measurements were within normal levels (Bell, 1993). A more likely explanation is that fetal blood glucose measurements were affected by delays in sampling fetal blood. Fetal glucose uptake may continue for some time after uterine blood flow stops, and the observed low fetal blood glucose levels may reflect acute decreases in fetal blood glucose due to ongoing fetal carbohydrate metabolism during the collection procedure. Amniotic fluid is relatively protected from such acute changes, and may therefore provide a more accurate indication of fetal glycemic status. In the current study, amniotic fluid glucose concentrations ranged between 1.6 mM (d 45) and 0.25 mM (d 138), suggesting that in vivo fetal blood glucose concentrations were higher than measured, and therefore more similar to previously published concentration data.

After glucose, lactate is quantitatively the next most important carbohydrate fuel in the fetus (Fowden, 1994). The only significant change observed in lactate levels was an increase in fetal blood lactate concentrations at d 138. The placenta is a rich source of lactate (Aldoretta et al., 1994) that is released into both uterine and umbilical circulations (Battaglia, 1978). Under hypoglycemic conditions placental lactate is preferentially partitioned into the fetal circulation (Aldoretta and Hay, 1999; Carver and Hay, 1995). Lactate is also produced by the fetus under normal conditions (primarily in skeletal muscle) (Hay, 1995b), and the rate of fetal lactate production increases with fetal hypoxemia (Hooper, 1995). The late gestation rise in fetal blood lactate concentrations may reflect normal fetal adaptation to an increasingly limited maternal nutrient/oxygen supply.
4.4.2 Ovine placental GLUT1 and GLUT3 expression studies

4.4.2.1 Northern analysis of oGLUT1 and oGLUT3 mRNA abundance

Northern analysis of placental total RNA showed ovine placental GLUT1 gene expression increased rapidly during the first trimester to a peak at d 120 of gestation. Abundance of mRNA then decreased to approximately 70% of the maximal level by d 138. In contrast, oGLUT3 gene expression continued to rise throughout gestation. These ontogenic changes in placental GLUT gene expression may reflect developmental changes occurring in the placenta. The pattern of oGLUT1 gene expression is similar to that seen for placental growth (defined by wet weight), while oGLUT3 gene expression parallels the continuous maturation of placental function throughout gestation. These gestational changes in placental GLUT gene expression are similar to those found in the developing rat placenta. Zhou and Bondy (1993) reported high levels of GLUT1 mRNA in the junctional zone during the mid gestational period of rapid growth, followed by a decline in term placenta. In rats, the relative intensity of placental GLUT3 gene expression per unit area did not change over gestation. However, the surface area of the labyrinthine zone, which demonstrates GLUT3 gene expression, increased greatly during the course of gestation, leading to an overall increase in placental GLUT3 mRNA abundance (Zhou and Bondy, 1993).

In the current study, relative abundance of both oGLUT1 and oGLUT3 appeared high in ovine placenta. In human placenta, both GLUT1 and GLUT3 mRNA have been reported in homogenates of term placenta (Bell et al., 1990). However, recent studies suggest that GLUT1 is the major isoform (Barros et al., 1995; Jansson et al., 1993), with GLUT3 gene expression low (Sakata et al., 1995), and protein levels low (Arnott et al., 1994; Haber et al., 1993; Shepherd et al., 1992) or undetectable (Barros et al., 1995; Jansson et al., 1995). This contrasts with the current study, which demonstrated oGLUT3 mRNA was abundant in ovine placenta. The variation in placental GLUT3 mRNA and protein abundance between species may reflect the differences in the morphology and physiology of human, rodent and ovine placentae (Dawes, 1968). In sheep, the transplacental glucose concentration gradient is much greater than in humans (Battaglia and Meschia, 1986), suggesting that the sheep placenta, with its discrete cotyledonary structure, has a lower placental permeability to glucose than the discoid hemochorial human placenta. The high relative abundance of GLUT3 gene expression we observed in ovine placenta may compensate for this low permeability.
4.4.2.2 Western analysis of oGLUT1 protein levels

In the current study, Western analysis of both total membrane and plasma membrane protein fractions from d 45, 90 and 138 placentomes showed oGLUT1 concentrations increased continually throughout gestation. This finding was supported by apparent increases in oGLUT1 immuno-signal observed in concurrent immunohistochemical studies. Furthermore, Western analysis of enriched plasma and internal membrane protein fractions demonstrated that the relative abundance of oGLUT1 protein per unit plasma (but not internal) membrane protein increased throughout gestation. This implies that the developmental increase seen in oGLUT1 protein abundance is not only due to increased membrane surface area, but may also reflect an increase in active GLUT1 protein concentration per unit area of plasma membrane.

The protein data contrasts with the apparent decrease observed in oGLUT1 mRNA abundance in late gestation placentomes by Northern analysis, and implies post-transcriptional regulation of oGLUT1. Other researchers have found no evidence for post-transcriptional regulation of placental oGLUT1 during gestation. Erhardt and Bell (1997) reported placental oGLUT1 mRNA and protein concentrations both increase from d 75 to d 110, and remain unchanged at d 140. In the current study, oGLUT1 mRNA abundance was not significantly different in d 90 and d 138 placentomes, and the mid to late gestation peak would not have been apparent without sampling d 120 gestation placentomes.

Post-transcriptional regulation of GLUT1 has been demonstrated in rat brain (Boado and Pardridge, 1993). However, further study is required to clarify developmental regulation of oGLUT1 protein in ovine placenta.
4.4.3 Ovine placental GLUT1 and GLUT3 localisation studies

ISH in late gestation placentomes (d 126) showed both oGLUT1 and oGLUT3 mRNA was abundant. Though present in maternal and fetal tissue, both were predominantly localised to the maternal-fetal barrier. In ovine placenta, uterine epithelium loses its cellular organisation and forms a partial syncytium that appears to lack definite basement membrane (Steven, 1975; Wooding et al., 1992). Fetal binucleate cells (BNCs) migrate and merge with maternal cells, modifying the uterine epithelium into a hybrid feto-maternal syncytiotrophoblast layer. In contrast, the fetal chorionic epithelial cells retain their cellular structure, and are cuboidal in shape with large nuclei (Wooding et al., 1992). Limitations of ISH and light microscopy made accurate cellular and sub-cellular localisation difficult. However, in the current study, oGLUT1 hybridisation signal in late gestation (d 126) ovine placenta appeared to be predominantly localised directly over clusters of multiple nuclei, suggesting localisation to both sides of the maternal syncytiotrophoblast, whereas oGLUT3 mRNA appeared to be localised to the fetal-facing side of the syncytiotrophoblast membranes.

Strong oGLUT1 hybridisation signal was evident in late gestation (d 126) endometrial tissue, and at the maternal-facing surface of maternal primary villi emerging from the endometrium. This oGLUT1 mRNA hybridisation signal was not prevented by either RNase A pretreatment or by competitive hybridisation controls, raising questions about probe specificity. However, the same regions were strongly immuno-positive for oGLUT1 protein using immunohistochemistry, with oGLUT1 immuno-signal absent in NRS and peptide preadsorbed control sections. In rats (Zhou and Bondy, 1993) and humans (Jansson et al., 1993) GLUT1 is predominantly located on maternal-facing membranes, and the proximal maternal villi are areas where high levels of glucose transport may be expected. Further ISH experiments using sense oGLUT1 oligonucleotide control probes are needed to validate the specificity of this intense oGLUT1 mRNA hybridisation signal.

Studies investigating the ontogeny of oGLUT localisation by ISH provided further evidence for distinct localisation of oGLUT1 and oGLUT3 mRNA. Ovine placental tissues originally collected for ISH were found to be unsuitable following the fixation procedure, so placental tissues collected for RNA extraction were used. Snap freezing destroys cellular architecture, and these tissues were not ideal for ISH. However, preliminary observations in d 45, 60, and 90 placentomes confirmed oGLUT1 mRNA was present in both maternal and fetal (in
particular d 45) cells, but was most strongly localised to maternal syncytiotrophoblast and tissue, whereas oGLUT3 appeared most strongly localised to the fetal trophoblast.

Results from the current study are similar to previously published studies using rat (Zhou and Bondy, 1993) and human (Hauguel-de Mouzon et al., 1997; Jansson et al., 1995) placenta, and support the hypothesis that oGLUT1 transporters are responsible for glucose transport from mother to placenta, whereas oGLUT3 transports glucose between placenta and fetus (Zhou and Bondy, 1993). Recent studies in humans have shown GLUT3 mRNA is widespread in villous tissue (Hauguel-de Mouzon et al., 1997; Jansson et al., 1995), but GLUT3 protein is restricted to fetal arterial vascular endothelium (Hauguel-de Mouzon et al., 1997; Head et al., 1999). Human placental GLUT distribution differs from that in other mammalian species (Illsley, 2000a). However, these data underline the need for caution when extrapolating from mRNA to protein distribution, and highlight the need for oGLUT3 protein localisation studies.

In the current study, immunohistochemical studies revealed that oGLUT1 protein localisation not only differed from observed oGLUT1 mRNA distribution, but also differed in localisation in early and late gestation. In contrast to mRNA expression, early gestation (d 45) oGLUT1 immuno-signal was low in maternal and fetal villous tissue, and immunopositive staining was restricted to fetal trophoblast cells and the invading trophoblast cells of the terminal fetal villi. As gestation progressed oGLUT1 immunopositive staining increased in maternal villous tissue. By d 90 the maternal villi showed strong immunopositive staining for GLUT1 and the intense immuno-signal in terminal fetal villi was lost. Such ontogenetic changes in oGLUT1 protein distribution may reflect placental growth and development. GLUT1 is the predominant GLUT isoform in fetal and embryonic tissue, suggesting it plays an important role in cell proliferation and tissue development (Asano et al., 1988). During early gestation the placenta undergoes rapid growth, and metabolically active mitotic trophoblast cells may be the primary glucose/energy consumers during rapid expansion of the fetal trophoblast. However, by d 90 placental DNA content is maximal (Ehrhardt and Bell, 1995), and maternal glucose is then required for placental metabolism and transport to the fetus.
4.5 SUMMARY AND FUTURE WORK

Northern analysis, using the oGLUT1 and oGLUT3 cDNAs generated in the study described in Chapter 3, demonstrated:

- Both oGLUT1 and oGLUT3 mRNA were abundant in ovine placenta.
- Both oGLUT1 and oGLUT3 mRNA were present from d 45 to d 138 (term d 145±2).
- Ovine GLUT1 and oGLUT3 showed distinct patterns of temporal gene expression during placental development. GLUT1 gene expression paralleled the changes seen in placental weight during gestation, suggesting oGLUT1 may play a role in maintaining glucose supply in early pregnancy during a period of rapid placental growth. In contrast, GLUT3 gene expression continued to increase throughout gestation. This pattern of development may reflect changes occurring during placental maturation.

Western analysis of oGLUT1 protein concentration demonstrated:

- A late gestation increase in oGLUT1 in contrast to the decrease observed in oGLUT1 mRNA abundance from mid to late gestation. This finding indicates late gestation post-transcriptional regulation of placental oGLUT1.
- Relative abundance of oGLUT1 protein increased in the plasma membrane fraction throughout gestation. This suggests that developmental increases in oGLUT1 protein may be due to increased oGLUT1 concentration per cell, as well as to an increase in the number of GLUT1 expressing cells during villous membrane expansion.

Localisation studies (ISH and immunohistochemistry) demonstrated:

- Ovine GLUT1 mRNA and protein, though present in both maternal and fetal cells, was predominantly localised to maternal syncytiotrophoblast and maternal villous tissue. In contrast, oGLUT3 mRNA was localised to fetal trophoblast and fetal-facing placental membranes.
- Although oGLUT1 protein was predominantly localised to maternal villous tissue in late gestation (d 126), concentrations were high in (and restricted to) fetal trophoblast cells during early gestation (d 45).

A major limitation of this study was the lack of oGLUT3 protein data, and development of an ovine specific GLUT3 antibody is essential for future oGLUT3 protein expression and localisation studies.
Further studies are needed to clarify ovine placental GLUT localisation.

- Future ISH studies should include sense oligonucleotide probe negative controls.
- The ISH ontogeny pilot study needs to be repeated and expanded using fresh frozen placentomes with intact cellular architecture.
- Cellular localisation should be verified by including immunohistochemical markers for structural orientation. For example, anti-cytokeratin distinguishes epithelial structures such as cyto- and syncytiotrophoblasts, and is useful for discrimination between extravillous cytotrophoblast and decidual cells, or intra-arterial trophoblast and maternal endothelium. Anti-vimentin stains all mesenchyme-derived cells, and is also useful for distinguishing decidual cells and maternal endothelium from trophoblast cells. Anti-factor VIII (the former anti-Von Willibrand factor) is a specific marker for most endothelial cells, and monoclonal MIB-1 may be used to stain proliferating cells. This approach is limited by the availability of ovine specific, or ovine cross-reactive, antibodies.
CHAPTER 5

REGULATION OF PLACENTAL
oGLUT1 AND oGLUT3 GENE EXPRESSION

BY LATE GESTATION

MATERNAL UNDERNUTRITION AND GLYCEMIA
5.1 INTRODUCTION

Glucose is a major component of fetal nutrition during fetal growth (Battaglia and Meschia, 1978). It is the principle source of carbon for placental and fetal metabolism, and for tissue synthesis during fetal development (Battaglia and Meschia, 1986; Jones and Rolph, 1985). Under normal conditions, fetal glucose is supplied from maternal circulation by facilitative diffusion across the placenta (Hay, 1994b; Hay and Meznarich, 1990). Thus, transplacental glucose transport flux is determined by glucose concentration gradients between mother and fetus, and the number and activity of placental glucose transporter proteins (Johnson and Smith, 1980).

Altered maternal plasma glucose concentrations may lead to pathophysiological changes in the rate of placental glucose transport. Pregnancies complicated by nutritional intrauterine growth restriction (IUGR) and gestational (GDM) and insulin-dependent diabetes mellitus (IDDM) are characterised by abnormal levels of maternal and fetal glycemia, and are associated with altered placental and fetal growth and increased rates of congenital abnormality and perinatal mortality (Economides et al., 1991; Freinkel and Metzger, 1978; Gluckman and Harding, 1997b; Marconi et al., 1996; Nicolaides et al., 1989). In vitro experiments using perfused human placenta have shown that rates of placental glucose uptake and transfer to fetal circulation are positively correlated with glucose concentrations in the maternal perfusate (Hauguel et al., 1986). In vivo investigations of placental transfer in rats have confirmed that altered maternal glucose levels effect the rate of glucose flux to the conceptus (Thomas et al., 1990; Thomas and Lowy, 1992). However, the regulatory effects of maternal blood glucose concentration on placental glucose transporter expression and activity remain unclear.

The aim of the studies described in this chapter was to investigate the role of maternal blood glucose levels in regulating ovine placental glucose transport. We hypothesised that changes in maternal substrate levels and/or circulating blood glucose concentrations alter regulation of placental oGLUT gene expression. We therefore examined oGLUT1 and oGLUT3 gene expression in late gestation ovine placentomes collected from two studies designed to investigate the effects of altered maternal nutrient supply on fetal growth. The first study, carried out in collaboration with Professor Jane Harding (RCDMB, Auckland, NZ) investigated the effects of maternal undernutrition and subsequent refeeding on late gestation fetal metabolism and growth. The second study, in collaboration with Professor W.W. Hay, (University of Colorado, Denver, Colorado, USA) investigated the effects of maternal...
hypoglycemia (to model the effects of decreased maternal nutrient supply) and hyperglycemia (to model the effects of maternal diabetes) during late gestation.

These studies demonstrated that placental oGLUT1 and oGLUT3 gene expression is regulated differently by acute versus long-term maternal glycemia, and that placental oGLUT1 protein expression may be regulated at post-transcriptional level.
5.2 METHODS AND MATERIALS

5.2.1 Late gestation maternal undernutrition and refeeding

Placentomes and post-mortem data were collected for this experiment in collaboration with Professor Jane Harding (RCDMB, Auckland, NZ). Details of animal surgery and management may be found in Harding et al. (1997b) and Harding et al. (1997a).

Figure 5.1 illustrates the experimental protocol. Briefly, Coopworth-Border cross-bred ewes were maintained preconception and during early gestation in the field on as high a nutritional plane as possible, with supplementary food supplied. At d 95 gestation, ewes carrying singletons were brought into the laboratory and housed individually in metabolic cages with free access to water. They were fed *ad libitum* once daily with weighed quantities of concentrates (NRM Multifeed sheep nuts, NRM Feeds Limited, Auckland, NZ) and chopped barley straw. At d 105 gestation, the ewes were divided into three groups. A control group (Ad Lib) continued to be fed *ad libitum* (*n* = 6 analysed in the current study). The second group (UN10/RF10) was fed a restricted diet from d 105 - 114 of 30 g nuts and 100 g straw daily (pre-prandial maternal arterial blood glucose concentrations ~1.4 - 1.6 mM). On d 115 they were returned to *ad libitum* feeding (*n* = 6 analysed in the current study). The third group (UN20) was maintained on a restricted diet for 20 d. From d 105 - 114 they were fed 30 g
nuts and 100 g straw daily. From d 115 – 124 they were fed 50 g nuts and 100 g straw daily (n = 6 analysed in the current study).

Although the original study was primarily designed to investigate the postnatal consequences of intrauterine nutrition and development, a subset of animals was studied after post-mortem at d 125 of gestation. From these ewes, type A placentomes (n = 6 per experimental group) were sampled for the current study (see section 1.2.1.2 for placentome morphology classification). Tissue was snap frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

5.2.2 Late gestation maternal glycemia

This study was undertaken in collaboration with Professor William Hay (University of Colorado, Denver, Colorado, USA), who kindly provided placentomes and animal data. Experimental details may be found in Aldoretta et al. (1994), Das et al. (1998), Das et al. (1999), and Hay et al. (1989).

Briefly, late gestation Columbia-Rambouillet mixed breed pregnant ewes were chronically-catheterised under general anaesthesia during the last 2 – 5 weeks of pregnancy, then recovered for two days prior to study. Ewes were maintained in separate carts although housed together, and had ad libitum access to alfalfa pellets, mineral blocks, and water. One group of pregnant ewes served as controls and received saline infusion. The experimental groups received intravenous infusions of either insulin (pure porcine ‘regular’ insulin, Eli Lilly and Company, Indianapolis, Indiana, USA) sufficient to maintain maternal arterial plasma glucose concentrations at approximately 50 – 60% normal levels (~1.4 – 1.7 mM versus ~2.8 mM), or glucose (50% dextrose w/v in H₂O) sufficient to maintain maternal arterial plasma glucose concentrations at approximately twice normal levels (~5.5 mM versus ~2.8 mM). The infusions were adjusted to maintain maternal hypo- or hyperglycemia according to once or twice daily measurements of maternal arterial plasma glucose concentrations.

For the current study, sample selection was based on recorded animal data, and was designed to select placentomes from singleton, late gestation (between d 133 – 143) pregnancies, matched for duration of altered maternal glycemia. The hypoglycemic samples were selected from ewes which had received insulin infusions for 48 h, 7 – 14 d, and 34 – 37 d (n = 4 per group). The hyperglycemic samples were selected from ewes that had received glucose
infusions for 3 – 4 h, 24 – 48 h, 6 – 9 d, and 18 – 19 d (n = 4 per group). The same control samples (n = 4) were used for both hypoglycemia and hyperglycemia studies. Frozen tissue was processed for RNA extraction (section 2.3.2.1), and plasma, internal and total post-nuclear membrane protein fractions were prepared as described in section 2.4.2.1.
Chapter 5

5.3 RESULTS

5.3.1 Late gestation maternal undernutrition

5.3.1.1 Post-mortem data
Analysis of post-mortem data from animals used in the current study showed no significant differences in fetal weight (p = 0.2), total placentome weight (p = 0.26), or fetal to placental weight ratio (p = 0.14) among groups (Figure 5.2). However, average placentome weight (Figure 5.3 A) decreased (p = 0.05), and average placentome number per placenta (Figure 5.3 B) increased in UN20 ewes (p = 0.005).

5.3.1.2 Placental oGLUT1 and oGLUT3 gene expression
Combined densitometric quantitation data from triplicate Northern analyses demonstrated no significant differences in the relative abundance of either oGLUT1 (p = 0.23) or oGLUT3 (p = 0.23) mRNA in placentomes from Ad Lib, UN10/RF10, or UN20 animals (Figure 5.4 A).

5.3.2 Late gestation maternal glycemia

5.3.2.1 Placental oGLUT1 and oGLUT3 gene expression
In hypoglycemic ewes (Figure 5.5 A), mean densitometric values for oGLUT1 and oGLUT3 mRNA levels, although elevated, were not significantly different from control values after 24 – 48 h. The relative abundance of oGLUT1 mRNA showed a greater than 2-fold decrease between 24 – 48 h, and 6 – 15 d and 34 – 36 d (p < 0.0001), although mean oGLUT1 mRNA levels at 6 – 15 d and 34 – 36 d were not significantly different from mean control values. Similarly, mean oGLUT3 mRNA levels decreased more than 2-fold in ewes hypoglycemic for 34 – 36 d when compared to ewes hypoglycemic for 24 – 48 h (p < 0.017), although mean oGLUT3 mRNA abundance was not significantly different from mean control values at any time-point measured. Placentomes from ewes with acute hypoglycemia (3 – 8 h) were not available for study.

In hyperglycemic ewes (Figure 5.6 A), the relative abundance of both placental oGLUT1 (p < 0.0001) and oGLUT3 (p < 0.0001) mRNA was significantly greater in ewes subjected to acute (3 – 8 h) maternal hyperglycemia than in control animals or ewes hyperglycemic for 24 – 48 h, 6 – 9 d or 15 – 19 d. Acute maternal hyperglycemia (3 – 8 h) caused a greater than 2.5-fold increase in the relative abundance of oGLUT1 mRNA, and a greater than 4-fold increase
in the relative abundance of oGLUT3 mRNA. By 24 – 48 h both oGLUT1 and oGLUT3 mRNA levels had returned to levels similar to those observed in control animals, and showed no further significant differences at 6 – 9 d, or 15 – 19 d.

5.3.2.2 Placental oGLUT1 protein levels

In hypoglycemic ewes (Figure 5.7 A), an increase of approximately 60% was observed in mean placental oGLUT1 protein levels after 48 h maternal insulin infusion (p = 0.002). However, after 7 – 10 d maternal hypoglycemia, mean oGLUT1 protein levels had returned to basal levels, and were not significantly different from mean control oGLUT1 densitometric values.

Maternal hyperglycemia did not immediately affect placental oGLUT1 protein levels, with no significant changes observed after acute (3 – 4 h) or short-term (24 – 48 h) maternal hyperglycemia (Figure 5.8 A). However, chronic maternal hyperglycemia (8 – 9 d) resulted in an approximately 70% increase in placental oGLUT1 protein levels compared with those of control animals (p = 0.0002).
Figure 5.2 A Fetal weight, B total placentome weight, and C fetal:placental weight data recorded at post-mortem for Ad Lib (n = 6), UN10/RF10 (n = 6) and UN20 (n = 6) ewes. Data is expressed as mean +/- SEM. No significant differences were observed among groups (fetal weight, p = 0.20; total placentome weight, p = 0.26; fetal:placental weight, p = 0.14, ANOVA).
Figure 5.3 A Mean placentome weight and B number of placentomes per placenta in Ad Lib (n = 6), UN10/RF10 (n = 6) and UN20 (n = 6) ewes. Data is expressed as mean ± SEM. Different letters denote significant differences (*p = 0.05, **p = 0.005, ANOVA/Bonferroni Dunn).
Figure 5.4 A Densitometric quantitation analysis of placental oGLUT1 and oGLUT3 mRNA in Ad Lib, UN10/RF10, and UN20 ewes. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean Ad Lib value. Graphs present mean percentage values ± SEM from triplicate Northern analyses. No significant differences were observed (p = 0.23, p = 0.23, ANOVA). B Representative Northern blot demonstrating placental GLUT1 (8 - 10 kb and 2.8 kb) and GLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in placentomes from Ad Lib (n = 6), UN10/RF10 (n = 6) and UN20 (n = 6) ewes (20 μg total RNA loaded per sample).
Figure 5.5 A Densitometric quantitation analysis of placental oGLUT1 and oGLUT3 mRNA in hypoglycemic ewes. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values ± SEM from triplicate Northern analyses. Different letters denote significant differences (\( p < 0.0001 \), \( p < 0.017 \), ANOVA/Bonferroni Dunn). B Representative Northern blot demonstrating placental oGLUT1 (8 - 10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in control animals (n = 4), and in ewes rendered hypoglycemic (-1.4 - 1.7 versus -2.8 mM) for 24 - 48 h (n = 4), 6 - 15 d (n = 4) or 34 - 36 d (n = 4) (20 μg total RNA loaded per sample).
Figure 5.6 A Densitometric quantitation analysis of placental oGLUT1 and oGLUT3 mRNA in hyperglycemic ewes. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values ± SEM from triplicate Northern analyses. Different letters denote significant differences (\(^{a}p < 0.0001, \quad ^{b}p < 0.0001, \quad \text{ANOVA/Bonferroni Dunn})

B Representative Northern blot demonstrating placental oGLUT1 (8 - 10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in control animals (n = 4), and in ewes rendered hyperglycemic (~5.5 versus ~2.8 mM) for 3 - 8 h (n = 4), 24 - 48 h (n = 4), 6 - 9 d (n = 4) or 15 - 19 d (n = 4) (20 μg total RNA loaded per sample).
A Densitometric quantitation analysis of placental oGLUT1 protein in control ewes, and in ewes rendered hypoglycemic for 48 h or 7 - 10 d. GLUT1 band optical density was calculated as percentage of mean control value. Graph presents mean percentage values ± SEM from triplicate Western blots. Different letters denote significant differences ("p = 0.002, ANOVA/Bonferroni Dunn). B Representative Western analysis. (i) Three replicate Western blots were incubated with rabbit anti-mouse/rat GLUT1 polyclonal antibody (1:5000, 1:5000). (ii) A fourth replicate Western was incubated with NRS (1:5000, 1:5000) to control for non-specific binding.
Figure 5.8 Western analysis showing relative expression of immunoreactive GLUT1 in mid-late gestation ovine placenta after maternal hyperglycemia. Aliquots (1.5 μg) of deglycosylated total membrane protein were electrophoresed on 7.5% SDS-PAGE gels and transferred to membrane for immunoblotting.

A Densitometric quantitation analysis of placental oGLUT1 protein in control ewes, and in ewes rendered hyperglycemic for 3-4 h, 24-48 h or 8-9 d. GLUT1 band optical density was calculated as percentage of mean control value. Graph presents mean percentage values ± SEM from triplicate Western blots. Different letters denote significant differences (p = 0.0002, ANOVA/Bonferroni Dunn).

B Representative Western analysis. (i) Three replicate Western blots were incubated with rabbit anti-mouse/rat GLUT1 polyclonal antibody (1:5000, 1:5000). (ii) A fourth replicate Western was incubated with NRS (1:5000, 1:5000) to control for non-specific binding.
5.4 DISCUSSION

Animal studies employing *in vivo* tracer methodology have demonstrated changes in the rate of transplacental glucose flux with altered maternal glucose concentrations (Thomas and Lowy, 1992). However, the evidence for associated changes in glucose transporter activity or expression is still equivocal. To investigate the role of maternal nutrient/glucose supply in regulating placental glucose transporter expression, relative abundance of oGLUT1 and oGLUT3 mRNA was measured in placentomes of ewes subjected to late gestation undernutrition or altered glycemia.

5.4.1 Late gestation maternal undernutrition model

5.4.1.1 Post-mortem data

Fetal growth is greatest, and therefore most demanding of maternal glucose supply, during late gestation (Battaglia, 1978; Schneider, 1996). Previous studies have demonstrated that fetal sheep respond to late gestation maternal undernutrition within 2 – 3 d by slowing their rate of growth, and fetal growth resumes if maternal refeeding resumes within 21 d (Harding and Johnston, 1995; Mellor and Matheson, 1979; Mellor and Murray, 1982). Furthermore, late gestation maternal hypoglycemia leads to reduction in placental weight (Aldoretta *et al.*, 1994; Carver and Hay, 1995; DiGiacomo and Hay, 1990c). In the current study, no significant changes occurred in fetal weight, aggregate placental weight or fetal to placental weight ratio, although mean UN20 fetal weight and UN20 fetal to placental weight ratio were both lower than mean Ad Lib values (by 17% and 11% respectively). Post-mortem weight data used in the current study was limited to the same subset of animals taken from the original experiment for Northern analysis (*n* = 6 per experimental group), and the small sample size may have prevented statistical data from reaching significance.

Analysis of individual placentomes revealed that although aggregate placentome weight was not significantly changed after UN20, a significant decrease in average placentome weight and concomitant increase in average placentome number per placenta occurred after 20 d of maternal undernutrition. Previous studies have shown that glucose supply to the uteroplacenta regulates glucose partitioning into oxidative and non-oxidative pathways. The decrease in average placentome size observed in the current study may reflect a decrease in non-oxidative processes such as production of placental tissue, storage molecules (glycogen) and exported products such as lactate and fructose (Aldoretta *et al.*, 1994; Aldoretta and Hay, 1999).
In most species, placental growth occurs in the first half of gestation (Battaglia and Meschia, 1986). In sheep, polycotyledonary placentation is fully established by about 30 days after conception, and the number of placentomes attached to each fetus is fixed at, or soon after, this time (Bell et al., 1999). Until mid gestation (d 75) hyperplastic growth increases placental weight, which then declines towards term due to tissue dehydration and placental remodelling (Ehrhardt and Bell, 1995). The significant increase in placentome number seen in the current study after late gestation maternal undernutrition (post d 105) implies that placentome development may occur at any stage of gestation, and raises several questions. Are new caruncles recruited to form placentomes, or is the growth of dormant/nascent placentomes triggered (ie, placental growth or placental remodelling)? What are the triggers for this process, and do they differ from those that occur during normal placental development?

A recent study has shown that maternal undernutrition in sheep over the period of rapid placental hyperplastic growth (d 28 – 77), followed by restoration of maternal nutrition for the reminder of gestation, causes a significant increase in average placentome number and placentome weight (Heasman et al., 1998). The increase in placentome weight was associated with compensatory overgrowth of the fetal component of the placentome, and Heasman et al. proposed that elevated fetal IGF-1 levels triggered fetal placental growth in response to a rise in late gestation fetal glucose plasma levels (Heasman et al., 1998).

### 5.4.1.2 Placental oGLUT1 and oGLUT3 gene expression

No significant changes were measured by Northern analysis in placental oGLUT1 or oGLUT3 mRNA abundance in isolated placentomes after either UN10/RF10 or UN20. That the decrease in average placentome size observed after UN20 was not associated with a decrease in oGLUT mRNA abundance per placentome suggests that the decrease in average placentome weight was not associated with a decrease in placentome membrane surface area or glucose transporter number. This supports previous findings that placental glucose transfer capacity does not change with reduced placental weight following chronic late gestation maternal glucose restriction (Aldoretta et al., 1994; DiGiacomo and Hay, 1990c; Hay, 1995b). However, the observed increase in placentome number after UN20 may in fact result in an overall increase in oGLUT mRNA abundance in the aggregate placenta. Although this would not influence placental glucose transport whilst uterine glucose supply remained limited, it would ensure an extremely rapid response to increased maternal blood glucose concentrations on maternal refeeding.
Studies in pregnant ewes have shown the development of fetal gluconeogenesis after 5 – 7 d late gestation maternal undernutrition in the sheep (DiGiacomo and Hay, 1989; Hay, 1994a). This raises fetal plasma glucose concentration relative to materno-placental concentration and redirects glucose flux back towards the placenta. This adaptation to chronic fetal hypoglycemia not only maintains fetal glucose metabolism, but also allows a greater proportion of the diminished uterine glucose supply to be retained for placental glucose metabolism (Hay, 1995a). The increase in placentome number observed in the current study after 20 d maternal undernutrition may increase the total placental surface area for exchange, thus maximising maternal-placental and fetal-placental glucose flux. In addition, the increase in placentome number may maintain critical placental mass for placental metabolism.

5.4.2 Maternal glycemia model

5.4.2.1 Maternal hypoglycemia

In the current study, samples from ewes with acute (3 – 8 h) hypoglycemia were not available for study, so information about acute placental oGLUT regulation by maternal hypoglycemia was not obtained. Similarly, no information is available for oGLUT1 protein levels after chronic (> 30 d) maternal hypoglycemia.

In spite of approximately 50% increases, the elevations in mean oGLUT1 and oGLUT3 mRNA abundance observed by Northern analysis after short-term (24 – 48 h) maternal hypoglycemia were not statistically significant. Western analysis of protein expression after a similar period of maternal hypoglycemia (48 h) demonstrated a significant increase in mean oGLUT1 protein levels. Chronic maternal hypoglycemia resulted in a significant decrease in both oGLUT1 and oGLUT3 mRNA levels in comparison to short-term (24 – 48 h) levels. However, placental oGLUT mRNA abundance after chronic maternal hypoglycemia (34 – 36 d) was not significantly different from mean control levels. Western analysis showed placental oGLUT1 protein levels returned to levels that were not significantly different from control values after 7 – 10 d of maternal hypoglycemia (chronic hypoglycemia data was not available).

Physiological data from the same study showed that insulin infusion induced maternal hypoglycemia, with maternal glucose levels about half normal and insulin concentrations averaging about twice normal. There was concomitant fetal hypoglycemia and hypoinsulinemia, with insulin concentrations average about 50% of normal (Das et al., 1998).
The similar temporal patterns of increased expression of oGLUT1 mRNA and protein with short-term (24 – 48 h) maternal hypoglycemia suggest that the same factor increased both transcription and translation. GLUT proteins belong to a group of genes acutely regulated by stress response (Baldwin et al., 1997), and both glucose deprivation and hypoxia have been reported to increase GLUT transcription rates acutely, independently and synergistically (Bruckner et al., 1999).

Das et al. (1998) reported that chronic (15 – 41 d) maternal hypoglycemia caused a 30 – 60% decrease in oGLUT1 protein levels from control levels. Although there are no data from the current study for oGLUT1 protein levels after 7 – 10 d of maternal hypoglycemia, mRNA expression was decreased after long-term hypoglycemia when compared to short-term (24 – 48 h) hypoglycemia. Studies in late gestation sheep have shown that chronic restriction of substrate supply result in chronic hypoglycemia and hypoxemia, and that plasma concentrations of cortisol, adrenocorticotropic hormone (ACTH), catecholamines and glucagon are high in growth restricted fetal sheep (Owens et al., 1989). Chronic hypoglycemia stimulates gluconeogenesis in late gestation fetuses (Hay, 1995a).

Stress stimuli, such as hypoglycemia, increase secretion of ACTH from the anterior pituitary (Ganong, 1987). ACTH stimulates the adrenal medulla to release rapid acting catecholamines (ie, epinephrine and norepinephrine), and the adrenal cortex to secrete glucocorticoids (predominantly cortisol in humans and sheep). Glucocorticoids affect both glucose and protein metabolism. They are gluconeogenic, and decrease peripheral glucose utilisation, increase peripheral protein catabolism, and increase hepatic uptake of amino acids and production of glucose and glycogen. However, their role is mainly permissive, facilitating the gluconeogenic actions of glucagon during hypoglycemia (Ganong, 1987). Hahn et al. (1999) have demonstrated that the synthetic glucocorticoid triamcinolone-acetonide strongly, and dose dependently, down regulates GLUT1 mRNA and protein expression in cultured human trophoblast cells and rat placenta. Similar, but less pronounced effects are seen with GLUT3 expression. Thus, elevated glucocorticoid (cortisol) levels in the hypoglycemic sheep fetus, in conjunction with glucagon, may cause the down regulation of oGLUT1 and oGLUT3 mRNA observed in the current experiment with long-term maternal (and fetal) hypoglycemia.

5.4.2.2 Maternal hyperglycemia

In the current study, large increases were observed in the relative abundance of both oGLUT1 (2.5-fold) and oGLUT3 (4-fold) mRNA after acute (3 – 8 h) maternal hyperglycemia. Relative abundance of both oGLUT1 and oGLUT3 mRNA returned to control levels by 24 – 48 h and
remained unchanged with long-term maternal hyperglycemia (6 – 9 d and 15 – 19 d). Ovine GLUT1 protein levels did not follow the same pattern as oGLUT1 mRNA. Western analysis demonstrated an increase in oGLUT1 levels compared to control values only after long-term (8 – 9 d) maternal glycemia, with no change in acute levels.

Chronic fetal hyperglycemia eventually down regulates fetal pancreatic insulin secretion, exaggerating fetal hyperglycemia and shifting the increased rate of uterine glucose uptake more into uteroplacental glucose consumption rather than transfer to the fetus (Hay, 1996). Increasingly, placental glucose metabolism is directed to non-oxidative pathways, and one consequence is an increase in placental lactate production with export to the maternal circulation (Hay, 1995b). Physiological data from this study demonstrated that maternal hyperglycemia induced fetal hyperglycemia and fetal hyperinsulinemia during the first 48 – 72 h of glucose infusions. However, by the end of the first week, despite persistent fetal hyperglycemia, fetal insulin concentrations returned to normal range (12 ± 2 μU/ml). Fetal oxygen levels were no different in the hyperglycemic group, and no changes were measured in pH or in catecholamine levels (Carver et al., 1995; Das et al., 1998). The rapid increases in relative abundance of both oGLUT1 and oGLUT3 mRNA with acute maternal hyperglycemia implies transcriptional regulation of placental oGLUTs by an early response to maternal hyperglycemia. High concentrations of glucose have been shown to decrease GLUT1 mRNA and protein expression (Hahn et al., 1998b; Hauguel-de Mouzon et al., 1994). However, maternal hyperglycemia leads to an initial maternal and fetal hyperinsulinemia with consequent elevation in IGF-1 levels. This raises the possibility that either insulin or IGF-1 is responsible for this first phase response.

The placenta has high concentrations of both insulin (Desoye et al., 1994) and IGF receptors (Abu-Amero et al., 1998; Lacroix et al., 1995), and at high concentrations insulin may act via the type-1 IGF receptor (de Pablo et al., 1990). Insulin has been shown to increase both GLUT1 and GLUT3 mRNA and protein expression in vitro (Taha et al., 1995). It shares with hypoxia the ability to induce the HIF-1alpha/ARNT transcription complex of GLUT1 and GLUT3 in various cell types (Zelzer et al., 1998). Furthermore, studies using trophoblast cells isolated from first trimester chorionic villi have demonstrated insulin-induced increases in hGLUT1 mRNA abundance and D-glucose transport within 12 h (Gordon et al., 1995). Other studies by the same group have shown that insulin enhances D-glucose transport in a dose responsive manner from 10 ng/ml concentration, and IGF-1 stimulates glucose uptake from 100 ng/ml. IGF-2 also stimulates glucose uptake in a dose responsive manner, but with much lower efficacy than either insulin or IGF-1 (Kniss et al., 1994). In sheep, maternal IGF-1 has
been shown to alter placental metabolism. Infusion of maternal IGF-1 increases placental lactate production, and partitioning of lactate to the fetus for oxidation (Liu et al., 1994). These data together suggest that either insulin or the insulin-like growth factors may be responsible for the acute increase in placental oGLUT1 and oGLUT3 mRNA levels observed in the current study.

The discrepancy between oGLUT1 mRNA and protein data supports a growing literature that reports GLUT protein production may be regulated at many levels including transcriptional, post-transcriptional, translational and post-translational stages (McGowan et al., 1995b). Long-term maternal hyperglycemia may lead to changes in placental GLUT mRNA transcript stability, which though unmeasurable by Northern blot analysis, may account for the trend towards an increase in oGLUT1 protein seen with chronic hyperglycemia. Ismail-Beigi's group has reported a bi-phasic response by GLUT1 mRNA to hypoxia, with hypoxia per se inducing rapid induction of GLUT1 mRNA transcription, and inhibition of oxidative phosphorylation increasing GLUT1 mRNA stability and half-life (Behrooz and Ismail-Beigi, 1997; Shetty et al., 1993).

In the current study, it is unclear what factor(s) may be regulating the post-transcriptional increase observed in oGLUT1 protein levels with long-term maternal hyperglycemia. The weight of evidence suggests that high glucose levels usually suppress GLUT1 protein levels (Hahn et al., 1998b). One possibility is that increased maternal and fetal glucose levels lead to increased maternal and fetal IGF-1 levels, and IGF-1 up-regulates placental oGLUT1 protein expression (Maher et al., 1989; Maher and Harrison, 1990).

Das et al. (1998) reported that chronic maternal hyperglycemia caused an initial 3-fold increase in placental oGLUT1 protein levels by 48 h, followed by a persistent 40 – 50% decrease in oGLUT1 levels from 10 – 20 d. The reason for these contrasting results is not clear. However, sample size was small in both experiments (At 24 – 48 h; n = 2 (Das et al., 1998) and n = 3 in the current study).
5.5 SUMMARY AND FUTURE WORK

The studies in this chapter have shown that few changes may be observed in the relative abundance of placental oGLUT1 and oGLUT3 mRNA after long-term perturbation of maternal blood glucose concentrations. However, increased levels of placental oGLUT mRNA and protein measured after short-term changes in maternal glycemia demonstrated that important changes occur in transcriptional regulation of placental oGLUT mRNA within the first 24 h of altered maternal glycemia. In addition, the discrepancy between oGLUT1 mRNA and protein levels after long-term (6 – 10 d) maternal hyperglycemia suggests that post-transcriptional regulation also plays an important role. Finally, these studies have confirmed that the placenta itself adapts to compensate for restricted maternal nutrition. The increase in placentome number observed after long-term maternal undernutrition may lead to an overall increase in placental surface area. This may provide a mechanism for rapid plento-fetal glucose uptake upon maternal refeeding.

To gain a better understanding of acute placental oGLUT gene regulation it would be desirable to study the following:

- Samples from ewes with acute hypoglycemia to determine whether this too has important regulatory effects.
- Samples at several time-points within the first 24 h to investigate more fully the regulatory changes occurring with acute perturbations in maternal nutrient levels.

The factors that regulate placental GLUTs during maternal nutrition need to be isolated and studied. Other studies have shown that glucocorticoids (Hahn et al., 1999), IGF-1 and insulin (Gordon et al., 1995; Kniss et al., 1994) regulate GLUT1 and GLUT3 in trophoblast cells and rodent placentae. Therefore, we need to:

- Infuse cortisol, insulin and IGF-1 and measure their effects on placental GLUT expression.

Northern and Western analysis of total mRNA and protein are not sufficient to measure post-transcriptional regulatory processes. Relatively small changes in at each level of regulation may result in a large increase in glucose transport (eg, a 5-fold increase in transcription, a 5-fold increase in half life of mRNA, and a 5-fold increase in protein half life can result in a 125-fold increase in the protein product of that gene (Tsai et al., 1998).
For a greater understanding of placental oGLUT regulation, we need to investigate:

- Relative abundance of mRNA using Northern analysis or RNase protection assay.
- mRNA stability and altered mRNA half life using Nuclear run-on assays.
- Transcription factors and cytosolic binding proteins that alter mRNA transcription and stability.
- Active protein levels at the plasma membrane.

We need to know what is happening to placental oGLUT3 protein. GLUT1 is the constitutive GLUT, found on both sides of the rat (Zhou and Bondy, 1993) and human (Jansson et al., 1993) placenta, and responsible for basal glucose transport. GLUT3 is situated on the fetal side of the placenta in rats (Boileau et al., 1995; Zhou and Bondy, 1993) and work in this thesis suggests oGLUT3 may also be fetal-facing in ovine placenta (Chapter 4). GLUT3 is the most efficient of glucose transporters (Bell et al., 1990) and is therefore highly suited to transporting at the relatively low glucose concentrations that exist between placenta and fetus. It may well play the most important role in partitioning glucose between placenta and fetus, and we need to know its locality and abundance before we can understand placental glucose transfer. Therefore we need:

- An ovine specific anti-GLUT3 antibody to undertake oGLUT3 protein abundance and localisation studies.
CHAPTER 6

REGULATION OF PLACENTAL
oGLUT1 AND oGLUT3 GENE EXPRESSION
BY LATE GESTATION
FETAL GROWTH HORMONE (GH)
AND
INSULIN-LIKE GROWTH FACTOR-1 (IGF-1)
6.1 INTRODUCTION

Although the principal determinant of fetal growth is the nutrient supply available for fetal metabolism and tissue accretion, complex endocrine systems control both the provision of nutrients to the fetus and the fetal response to nutrient supply. Maternal, placental, and fetal endocrine systems interact to regulate fetal growth. Previously, the fetus has been regarded as a simple end user in this nutrient supply system. However, recent evidence has indicated that the fetus may in part direct its own nutrient supply, influencing the partitioning of nutrients between placenta and fetus by endocrine regulation of placental nutrient metabolism (Gluckman, 1995; Hay, 1994a). In vivo studies in late gestation fetal sheep have demonstrated that short-term infusion of insulin-like growth factor-1 (IGF-1) into the fetal circulation increases substrate uptake by the fetus and inhibits placental lactate production (Harding et al., 1994). These data suggest that in addition to direct anabolic effects on fetal growth, fetal IGF-1 may indirectly promote fetal growth by regulating placental function to enhance placental nutrient transfer to the fetus.

The studies described in this chapter were directed towards investigating fetal endocrine regulation of placental glucose transport. In particular, studies focused on the regulatory effects of fetal growth hormone (GH) and IGF-1 on placental oGLUT1 and oGLUT3 mRNA abundance. We hypothesised that fetal IGF-1, but not fetal GH, regulates placental GLUT1 and GLUT3 gene expression. To test this hypothesis we examined oGLUT1 and oGLUT3 gene expression in ovine placentomes collected at post-mortem from three studies designed to investigate the effects of late gestation fetal GH or IGF-1 infusions on fetal growth. The first study (Dr Michael Bauer, RCDMB, Auckland, NZ) investigated the effects of long-term fetal GH infusions on late gestation placental and fetal growth. The second study (Dr Julie Owens, Adelaide University, Adelaide, Australia) was designed to investigate the effects of long-term fetal IGF-1 infusion on fetal growth and skeletal maturation. The third study (Dr Ellen Jensen and Professor Jane Harding, RCDMB, Auckland, NZ) investigated the growth promoting effects of acute fetal IGF-1 infusions in normal fetuses and in fetuses in which placental nutrient transfer capacity had been reduced by experimentally induced placental embolisation.

These studies demonstrated no long-term effect of fetal GH or IGF-1 infusions on oGLUT1 or oGLUT3 gene expression in normal fetuses. In contrast, acute administration of IGF-1 resulted in significantly different levels of placental oGLUT3 (but not oGLUT1) mRNA.
abundance in normal and embolised fetuses. This may indicate that fetal IGF-1 is capable of regulating placental oGLUT3 gene expression. However, further studies are required to elucidate the role of fetal IGF-1 in regulation of placental glucose transport and metabolism.
6.2 METHODS AND MATERIALS

6.2.1 Fetal GH infusion

Placentomes and post-mortem data were collected for this experiment with kind permission from Dr Michael Bauer (RCDMB, Auckland, NZ). The current study was restricted to two experimental groups: a vehicle infused control group, and a group of fetuses with normal endogenous GH levels supplemented to approximately twice the normal level by experimentally infused GH.

Briefly, pregnant ewes (Coopworth/Romney/Dorset cross) were housed in individual cages three days prior to surgery. The ewes had ad libitum access to water and were fed a standard diet of lucerne hay and pellets daily. At d 115 of gestation (term d 145 ± 2), multiple catheters were surgically implanted into maternal and fetal arteries and veins.

Infusions were administered into the fetal femoral vein as soon as the animals recovered consciousness post surgery. Recombinant bGH (American Cyanamid, Princeton, NJ, USA) was dissolved in vehicle (0.01M carbonate buffered saline pH 9.4) to a concentration of 100 µg/ml. Control animals (n = 7 analysed in the current experiment) were infused with vehicle (0.01 M CBS) for 11 d. Experimental fetuses (n = 6 analysed in the current experiment) were infused with ~3.5 mg rbGH/d (~1.2 mg/kgBW based on d 120 fetal weight), which was calculated to approximately double circulating fetal GH concentrations. To mimic the normal pulsatile release pattern of GH, two GH infusions were co-administered: a constant infusion (2.4 mg/day at 100 µg/ml/h), and 30 superimposed pulse infusions per day (adding an additional ~1.1 mg/d).

On d 126 of gestation ewes were killed by phenobarbitone overdose. For the current study, placentomes were collected from each placenta, weighed, snap frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

6.2.2 Long-term fetal IGF-1 infusion

Selected placentomes and associated placentome weight data were kindly provided for this experiment by Dr Julie Owens (Adelaide University, Adelaide, Australia). Details of the original experiment may be found in Lok et al. (1996).
Briefly, pregnant Merino ewes of known gestational age were housed in individual cages one week prior to surgery. The sheep were fed alfalfa hay, oats and water daily *ad libitum*. At d 110 of gestation (term d 145 ± 2) multiple catheters were surgically implanted into maternal and fetal arteries and veins. Prophylactic antibiotics were administered for 3 d post-surgery, and maternal and fetal health were monitored by analysing blood gases every 2 d. Animals were given 10 d to recover following surgery, during which time they were assigned to either a vehicle treated control group (n = 8 used in the current study), or IGF-1 treated experimental group (n = 9 used in the current study). Ewes with singleton or twin pregnancies were selected in equal numbers for each group.

Treatment started on d 120 of gestation. Recombinant human IGF-1 (Gropep, Adelaide, Australia) was dissolved in vehicle (0.9% sterile saline containing 10 g/l bovine serum albumin, RIA grade – IGF and IGFBP free [Sigma, St Louis, MO, USA]) to a concentration of 0.42 mg/ml. Recombinant hIGF-1 (26 ± 3 μg.h⁻¹.kg⁻¹ based on d 130 fetal weight) or vehicle was infused continuously into the fetal tarsal vein catheter. On d 130 of gestation ewes were killed by phenobarbitone overdose. For the current study, one large and one small placentome was selected from each placenta, excised, weighed, snap frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.
6.2.3 Placental embolisation and acute fetal IGF-1 infusion

Placentomes were collected for this experiment with kind permission from Dr Ellen Jensen and Professor Jane Harding (RCDMB, Auckland, NZ). The aim of the original experiment was twofold: firstly to induce intrauterine growth restriction (IUGR) by impairing placental function, and subsequently to determine whether IGF-1 administered to the fetus could effectively prevent or reverse the effects of the induced IUGR. The method used to impair placental function was gradual destruction of the uteroplacental vascular bed by microsphere injection into the uterine artery. Details of the original experiment may be found in Jensen et al. (1999).

Figure 6.1 outlines the experimental protocol. Briefly, pregnant (Coopworth/Romney/Dorset cross) ewes carrying single fetuses were housed in individual cages. Ewes had ad libitum access to water and a diet of chaffage and pelleted stock feed. At d 112 – 118 of gestation
multiple catheters were surgically implanted into maternal and fetal arteries and veins. The animals were allowed to recover for three days post surgery, during which time blood gases and fetal growth were monitored, and the animals received prophylactic antibiotics daily.

Control ewes (n = 3 in the current study) and normal ewes (N+IGF-1, n = 6 in the current study) were given 2 ml sterile saline into both uterine arteries twice a day for seven days. To experimentally induce placental damage, ewes in the embolised group (E+IGF-1, n = 6 in the current study) were given 2 ml (3 x 10^5) of 50 µm diameter microspheres (New England Nuclear, Life Science Products, Boston, MA, USA) to each uterine artery twice a day for approximately 7 d. Daily fetal blood gas measurements were monitored to determine the effect of placental embolisation. If fetal arterial PO2 was below 15 mmHg or fetal lactate above 2.0 mM, 50 µ spheres were replaced with 2 ml (2 x 10^6) 15 µ spheres. Embolisation was stopped when implanted fetal growth catheters indicated fetal growth had slowed or stopped for several days.

Ten to 14 d after surgery (−d 126 – 129) a single IGF-1 challenge was administered to each experimental animal. A compound tracer infusion comprising antipyrine (250 mg), ^14^C-urea (250 µCi), and 3-O-[methyl-^3^H] D-glucose (1.5 mCi) was administered via the fetal femoral vein at 3 ml/h for 7.5 h to measure placental substrate transfer rates (figure 6.1). A separate infusion of saline (3 ml/h) administered via the same catheter was changed to IGF-1 infusion (50 µg/h/kg estimated fetal weight) from 3.5 – 7.5 h (control animals received saline infusion only). A set of five blood samples was taken at 15 min intervals at 2.5 h, and a second set of five blood samples was taken 3 h later over the last hour of the 4 h IGF-1 infusion. Immediately after the IGF-1 infusion, animals were killed by overdose of phenobarbitone. For the current experiment, selected placentomes were categorised, weighed, snap frozen in liquid nitrogen, and stored at −80°C for subsequent RNA extraction.

6.2.4 Statistical analysis

Unpaired Student’s t-test comparisons were used to analyse data, which are expressed as mean ± SEM.
6.3 RESULTS

6.3.1 Fetal GH infusion

6.3.1.1 Fetal and placental weight data
Measurements taken at post-mortem showed no difference in fetal weight between control (n = 7) and GH (n = 6) infused fetuses (Figure 6.2 A, p = 0.85). Similarly, no statistical differences were observed between control and GH infused fetuses in membrane weight (p = 0.93), uterus weight (p = 0.70), total placentome number (p = 0.68), total placentome weight (p = 0.88), or average placentome weight (p = 0.71) (Figure 6.2 B – F).

6.3.1.2 Placental oGLUT1 and oGLUT3 gene expression
Combined densitometric quantitation data from triplicate Northern analyses demonstrated no significant differences in the relative abundance of either oGLUT1 (p = 0.82) or oGLUT3 (p = 0.53) mRNA in placentomes from control and GH treated fetuses (Figure 6.3 A).

6.3.2 Long-term fetal IGF-1 infusion

6.3.2.1 Placentome weight and morphology
In the current study, one small and one large placentome was collected from each placenta, and these two groups were analysed separately. The average weight of both small (Figure 6.4 A) and large (Figure 6.4 B) placentomes was lower in IGF-1 infused than in control fetuses (#p = 0.08, *p = 0.04 respectively).

Placentomes used in this experiment were of mixed morphology, consisting of type A, B and C placentomes (see section 1.2.1.2 for classification of types). Both small and large placentome groups selected for the current study had a greater number of type B placentomes in control animals, and type A placentomes in IGF-1 treated animals (Table 6.1).
Table 6.1 Distribution of placentome types in control and IGF-1 treated animals

<table>
<thead>
<tr>
<th>Placentome type</th>
<th>Small placentomes</th>
<th>Large placentomes</th>
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<tr>
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<td>Control</td>
<td>IGF-1</td>
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<td>C</td>
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6.3.2.2 Placental oGLUT1 and oGLUT3 gene expression

Relative abundance of both oGLUT1 and oGLUT3 mRNA was lower in small placentomes than in large placentomes (cf, representative Northern blots in Figures 6.5 B and 6.6 B). In small placentomes, combined densitometric quantitation data from triplicate Northern analyses demonstrated significant decreases in relative abundance of both oGLUT1 (***p = 0.005) and oGLUT3 (***p = 0.0009, where asterisks indicate level of significance) mRNA in IGF-1 infused fetuses (Figure 6.5 A). In large placentomes (Figure 6.6 A) the decrease in oGLUT1 expression was not significant (p = 0.11), although the relative abundance of oGLUT1 mRNA appeared lower in IGF-1 infused than control fetuses. However, oGLUT3 mRNA levels were significantly decreased from control values in IGF-1 infused fetuses (*p = 0.04).

6.3.3 Placental embolisation and acute fetal IGF-1 infusion

6.3.3.1 Placental oGLUT1 and oGLUT3 gene expression

RNA extracted from placentomes collected from three control animals (saline infusion only) was degraded (data not shown), and could not be used for Northern analysis. Combined densitometric quantitation data from duplicate Northern analyses (Figure 6.7 A) demonstrated no significant difference (p = 0.24) in the relative abundance of placental oGLUT1 mRNA in normal + IGF-1 (N+IGF-1) compared with embolised + IGF-1 (E+IGF-1) treated fetuses. In contrast, oGLUT3 mRNA abundance was significantly higher in E+IGF-1 compared to N+IGF-1 fetuses (**p = 0.01).
Figure 6.2 Fetal and placental weight data recorded at post-mortem for control (n = 7) and GH treated (n = 6) fetuses. Data is presented as mean +/- SEM. No significant differences were observed (p < 0.05, Student’s t-test).
Figure 6.3 A Densitometric quantitation analysis of oGLUT1 and oGLUT3 mRNA in placenta from control and GH treated fetuses. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values +/-SEM from triplicate Northern analyses (p = 0.82, p = 0.53, Student’s t-test). B Representative Northern blot demonstrating placental oGLUT1 (8-10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in control (n = 7) and GH (n = 6) treated fetuses (20 μg total RNA loaded per sample).
Figure 6.4 A Small, and B large placenta weight data for control (n = 8) and IGF-1 (n = 9) treated fetuses. Data is expressed as mean ± SEM (#p = 0.08, *p = 0.04 where asterisk indicates significance, Student’s t-test).
**Figure 6.5** A Densitometric quantitation analysis of oGLUT1 and oGLUT3 mRNA in small placentomes from control and IGF-1 treated fetuses. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values +/- SEM from triplicate Northern analyses (**p = 0.005, ***p = 0.0009 where asterisks indicate level of significance, Student’s t-test). B Representative Northern blot demonstrating placental oGLUT1 (2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in small placentomes from control (n = 8) and IGF-1 (n = 9) treated fetuses (20 µg total RNA loaded per sample).
Figure 6.6 A Densitometric quantitation analysis of oGLUT1 and oGLUT3 mRNA in large placentomes from control and IGF-1 treated fetuses. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values +/- SEM from triplicate Northern analyses (p = 0.11, *p = 0.04 where asterisk indicates significance, Student’s t-test). B Representative Northern blot demonstrating placental oGLUT1 (8 - 10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in large placentomes from control (n = 8) and IGF-1 (n = 9) treated fetuses (20 μg total RNA loaded per sample).
Figure 6.7 A Densitometric quantitation analysis of oGLUT1 and oGLUT3 mRNA in normal+IGF-1 (N+IGF-1), and embolised+IGF-1 (E+IGF-1) infused fetuses. Glucose transporter band optical density after standardisation (mRNA/28S) was calculated as percentage of mean control value. Graphs present mean percentage values +/- SEM from duplicate Northern analyses (**p < 0.01 where asterisks indicate level of significance, Student's t-test). B Representative Northern blot demonstrating placental oGLUT1 (8-10 kb and 2.8 kb) and oGLUT3 (4.0 kb) mRNA transcripts and methylene blue stained 28S rRNA in placentomes from normal+IGF-1 (n = 6), and embolised+IGF-1 (n = 6) infused fetuses (20 µg total RNA loaded per sample).
6.4 DISCUSSION

The studies presented in this chapter provide no evidence for the regulation of placental oGLUT1 and oGLUT3 gene expression by long-term (10 – 11 d) administration of either GH or IGF-1 to the fetus. However, a pilot study investigating the effects of acute IGF-1 infusion (4 h) in normal fetuses and fetuses with placental embolisation demonstrated a significant difference in the relative abundance of placental oGLUT3 mRNA.

6.4.1 Long-term fetal GH infusion

Circulating levels of GH are high in the fetus (Gluckman, 1986), and GH receptor gene expression has been demonstrated in a variety of ovine tissues, including placenta (Hill et al., 1992; Klempt et al., 1993; Lacroix et al., 1996). Yet little is known about the effects of GH on fetal growth or placental nutrient transfer. In the original experiment from which placentomes were taken for the current study, physiological data indicated that long-term (11 d) infusion of GH to the sheep fetus promoted growth of major organs (Bauer et al., 1998). Furthermore, maternal GH infusion has been reported to increase simple and facilitative diffusion capacity in ovine placenta (Harding et al., 1997).

In the current study, GH infusion sufficient to approximately double circulating fetal GH levels for 11 d in late gestation did not alter fetal weight or any of the placental parameters studied. In accordance with the morphological data, neither oGLUT1 nor oGLUT3 gene expression differed from control values following long-term fetal GH infusion. These data provide no evidence to indicate that fetal GH affects placental growth or functional capacity to transport glucose. However, conclusions about the effects of fetal GH on placental glucose transport are limited by the fact that no data is available for placental glucose uptake, transfer or metabolism, placental GLUT protein expression, or placental GLUT mRNA or protein expression after acute fetal GH infusion.

6.4.2 Long-term fetal IGF-1 infusion

In mid to late gestation sheep, long-term (10 d) IGF-1 infusion promotes growth of major organs, endocrine glands and skeletal maturation (Lok et al., 1996). In the current study, data indicated that long-term fetal IGF-1 infusion caused significant decreases in average placentome weight, and in oGLUT1 and oGLUT3 mRNA abundance.
The decrease in weight observed for both small and large placentomes in the current study was not observed in placentomes from the original experiment (Lok et al., 1996). They found no effect of 10 d IGF-1 infusion on total placental weight, average placentome weight or number. However, they observed a change in size distribution of placentomes with an increase in the 12–13.9 g weight category in IGF-1 treated animals (measured weight range 0–28 g) (Lok et al., 1996). Large placentomes from the same size range used in the current study showed a significant decrease in weight with IGF-1 treatment.

Functional maturation of the placenta during late gestation is characterised by a number of physical changes to the placental barrier, including increased placental membrane area and decreased membrane diffusion distance (Owens, 1991). IGF-1 has been shown to promote early placental maturation and hasten development of the placental barrier (Owens, 1991). These observations taken together with results from the original experiment (Lok et al., 1996) suggest that the decrease in average placentome weight and placental GLUT mRNA levels observed in the current study are spurious. The current study was limited to one small and one large placentome selected from each placenta, and it is possible that small sample size and selection procedure biased both placentome weight data and measurement of placental oGLUT mRNA abundance.

6.4.3 Placental embolisation and acute fetal IGF-1 infusion

Analysis of Northern data showed no significant difference in placental oGLUT1 mRNA abundance between N+IGF-1 fetuses and E+IGF-1 fetuses. This is surprising in light of previous studies that have demonstrated a compensatory increase in placental nutrient transfer in response to reduced placental size (Owens et al., 1987c). In the current study, placental damage and experimental IUGR were induced by maternal administration of microspheres to the uterine arteries (Jensen et al., 1999). Previous studies have shown that chronic placental embolisation produces progressive hypoxia in late gestation fetal sheep (Gagnon et al., 1994), and in vitro and in vivo studies have demonstrated that hypoxia leads to an increase in GLUT1 mRNA abundance and protein biosynthesis [See Zhang et al., (1999) for review]. GLUT1 is predominantly localised to maternal facing placental membranes (Zhou and Bondy, 1993). It is possible that embolisation may have preferentially damaged maternal facing placental membranes, and masked any regulatory increase in placental oGLUT1 mRNA.
abundance that may have occurred in response to hypoxia by destroying GLUT1 mRNA containing cells.

Relative abundance of placental oGLUT3 mRNA was significantly greater in E+IGF-1 than N+IGF-1 fetuses. A major limitation to the current study was loss of control (normal fetuses with saline infusion) samples due to RNA degradation. This meant that, in the current experiment, the effects on placental oGLUT3 expression of acute fetal IGF-1 infusion could not be separated from the effects of placental embolisation. Therefore, the greater abundance of oGLUT3 mRNA in E+IGF-1 placentomes may reflect an increase in oGLUT3 mRNA abundance due to (a) placental embolisation or (b) acute infusion of IGF-1 following placental embolisation. Alternatively, this result may be interpreted as (c) a decrease in oGLUT3 mRNA abundance due to acute IGF-1 infusion in the N+IGF-1 fetus. The following sections explore each of these possibilities.

6.4.3.1 Argument for up-regulation of placental oGLUT3 mRNA expression in E+IGF-1 animals due to placental embolisation

Placental embolisation restricts substrate supply to the fetus (Jensen et al., 1999), and oGLUT3 gene expression may be up-regulated in response to low fetal glucose and/or oxygen concentrations in nutrient restricted fetuses. Hypoxic stress is known to up-regulate expression of both oGLUT1 and oGLUT3 via the hypoxia-inducible factor-1alpha (HIF-1alpha)/aryl hydrocarbon nuclear translocator (ARNT) (Zelzer et al., 1998), and hypoxia plus glucose deprivation synergize to markedly increase GLUT gene expression (Bruckner et al., 1999). Furthermore, reduced fetal nutrient and oxygen supplies cause an increase in the circulating concentrations of stress-related hormones such as adrenaline, noradrenaline, adrenocorticotropic hormone (ACTH) and cortisol (Hooper, 1995). These hormones are known to cause increases in cyclic AMP, which is a multifunctional ‘hunger signalling molecule’. Cyclic AMP activates protein kinases which in turn lead to increased expression in a number of proteins involved in energy flux, including GLUTs (Vinals et al., 1997).

6.4.3.2 Argument for up-regulation of placental oGLUT3 mRNA expression in E+IGF-1 animals by acute IGF-1 infusion

IGF-1 may up-regulate GLUT3 gene expression via the HIF-1alpha/ARNT (Zelzer et al., 1998) or via IGF-1 receptors (Kniss et al., 1994; Lacroix et al., 1995), raising the possibility that acute IGF-1 infusion increased oGLUT3 expression in E+IGF-1 fetuses. However, this is
unlikely because Jensen et al. (1999) demonstrated placental resistance to the metabolic effects of IGF-1 in growth restricted (E+IGF-1) fetuses.

Jensen et al. (1999) confirmed previous reports (Harding et al., 1994) that acute infusion of IGF-1 to normal sheep fetuses decreases fetal blood glucose, oxygen, urea and amino-nitrogen concentrations, and inhibits placental lactate production, indicating that IGF-1 partitions glucose to fetal utilisation rather than placental metabolism. They reported the same changes in fetal metabolites with E+IGF-1 fetuses, but the effect on placental lactate inhibition was not seen. Furthermore, in normal fetuses, IGF-1 infusion caused a fall in umbilical blood flow and placental clearance of 3-O-[methyl-3H] D-glucose (a measure of placental capacity to transport by facilitative diffusion). In E+IGF-1 animals, uterine and umbilical blood flow and placental 3-O-[methyl-3H] D-glucose clearance were low prior to IGF-1 infusion, and remained unaffected by acute fetal IGF-1 infusion (Jensen et al., 1999). Taken together these data suggest that embolised placentae are resistant to the effects of fetal IGF-1.

IUGR is characterised by multi-hormone resistance (Gluckman and Harding, 1997a). Resistance to IGF-1 may develop due to a decrease in receptor numbers or binding capacity (LeRoith et al., 1991), or due to altered levels or binding capacities of IGF binding proteins (IGFBPs), that alter the bioavailability of IGF-1 (Sara and Hall, 1990). In particular, an increase in plasma concentrations of IGFBP-1 has been associated with IUGR (Unterman et al., 1990). IGFBP-1 is acutely up-regulated by low circulating fetal glucose and insulin levels, and an inverse correlation has been demonstrated between fetal plasma IGFBP-1 concentrations and fetal growth (Lee et al., 1997). In the current study, high circulating IGFBP-1 in growth-restricted fetuses (E+IGF-1) may have acted to reduce the bioavailability of exogenous IGF-1 to placental tissue. Alternatively, placental embolisation may have led to low fetal plasma glucose concentrations, and low circulating fetal insulin and endogenous IGF-1 levels. Placental IGF-1 receptors may have been down-regulated as a consequence, and were unavailable to mediate the actions of infused IGF-1.

6.4.3.3 Argument for down-regulation of placental oGLUT3 mRNA expression in N+IGF-1 animals by acute IGF-1 infusion

Alternatively, acute IGF-1 infusion to the normal fetus may down-regulate placental oGLUT3 gene expression. Initially, this seems counter-intuitive to IGF-1 promoting fetal growth. However, acute regulation of fetal-facing placental GLUT expression may prevent
fetal glucose from being lost back to the placenta during post-prandial glucose supply surges. Furthermore, in the normal animal high fetal IGF-1 levels usually signal high fetal arterial glucose concentration, which is known to regulate the net transfer of glucose from the placenta to the fetus (Hay, 1995a). GLUT3 is localised to fetal-facing membranes (Hauguel-de Mouzon et al., 1997; Jansson et al., 1995; Zhou and Bondy, 1993) and may be downregulated as part of this process. Supporting this hypothesis, Jensen et al. (1999) reported acute IGF-1 infusion to normal fetuses caused decreased umbilical blood flow, a 40% decrease in placental clearance of 3-0-[methyl-3H] glucose, and a 45% decrease in placental lactate production.

Direct down-regulation of placental oGLUT3 by circulating IGF-1 is unlikely because IGF-1 is known to up-regulate GLUT3 gene transcription (Kniss et al., 1994; Zelzer et al., 1998). However, metabolic changes associated with acute fetal IGF-1 infusion may trigger the down-regulation of placental oGLUT3. Products of intermediary metabolism are known to regulate glucose transport. In heart and skeletal muscle carbohydrates (lactate and pyruvate), organic acids (propionate and acetate), ketone bodies (β-hydroxybutyrate), long-chain fatty acids (palmitate) and citric acid cycle intermediaries (citric acid, α-oxoglutarate) have all been shown to depress glucose transport, with lactate, pyruvate and propionate most potent (Fischer et al., 1997; Randle et al., 1994a; Randle et al., 1994b; Shubert et al., 1996). Jenson et al. (1999) reported that acute IGF-1 infusion to normal, but not embolised, fetuses increased fetal blood lactate concentrations by approximately 25%. Taken together, these observations indicate that lactate may be a candidate for regulation of placental GLUT3 gene expression.

If such acute regulation occurs, the rapid decrease in relative abundance of oGLUT3 mRNA is likely to be due to a decrease in mRNA stability rather than a decrease in mRNA transcription. Acute regulation may also involve other rapid regulatory mechanisms such as internalisation of GLUT3 protein at the cell surface (Hahn et al., 2000).
6.5 SUMMARY AND FUTURE WORK

The studies in this chapter provide no evidence to support direct regulation of placental oGLUT1 or oGLUT3 by long-term infusion of fetal GH or IGF-1. However, they indicate that placental embolisation and/or acute fetal IGF-1 may regulate placental GLUT gene expression, and have raised intriguing questions about fetal metabolic regulation of placental glucose transport, and placental-fetal carbohydrate interactions.

Long-term (10 – 11 d) infusion of exogenous GH or IGF-1 to the ovine fetus did not alter relative abundance of placental oGLUT mRNA. For a more complete understanding of placental GLUT regulation it would be desirable to study placental oGLUT1 and oGLUT3:

- mRNA and protein levels from fetuses treated with acute (0 – 4 h) and short-term (24 – 48 h) GH and IGF-1 infusions.
- mRNA transcription and degradation rates to investigate transcriptional regulation, and protein levels to investigate post-transcriptional GLUT regulation (ideally measure only activated placental GLUT protein at the cell surface).

Pilot study data from growth restricted and normal fetuses acutely (4 h) infused with IGF-1 have indicated a change in placental oGLUT3 mRNA abundance. However, it remains unclear as to whether reduced substrate supply or acute IGF-1 infusion caused such changes. To forward this study we need to study:

- Placentomes from fetuses infused with saline alone to determine placental oGLUT levels at normal fetal IGF-1 plasma concentrations.
- Placentomes from fetuses treated by placental embolisation alone to determine the effect of embolisation on placental oGLUT levels.

These studies intimate that fetal metabolism may alter placental oGLUT3 mRNA levels. However, much remains unclear, and further studies are required to define regulation and to separate the regulatory effects of IGF-1 and other metabolic and endocrine factors. Parameters that require further investigation are:

- Fetal and placental metabolite levels (lactate, pyruvate, propionate, amino acids, oxygen and glycogen).
- Placental IGF-1 receptor, and fetal circulating IGF-1 and IGFBP (particularly IGFBP-1) levels.
CHAPTER 7

CONCLUSIONS
7.1 SUMMARY

The main focus of this thesis was the examination of oGLUT1 and oGLUT3 gene expression during ovine placental development and function. Glucose is transported by facilitated diffusion (Johnson and Smith, 1980). Thus, placental glucose transport from mother to fetus may be affected by factors that alter the transplacental glucose concentration gradient, or by factors that directly regulate the number and/or activity of GLUT proteins present in placental membranes. Experimental models of pregnancy using chronic catheterisation and in vivo tracer methodology have elucidated many of the maternal, placental and fetal factors that alter transplacental glucose concentration gradients and direct the flux of glucose to the fetus during pregnancy (Harding and Johnston, 1995; Hay, 1995a; Owens, 1991). However, the molecular regulation of placental GLUT proteins, and the extent to which their regulation may influence fetal glucose supply remains unclear.

The sheep provides a good model for the study of placental glucose transport because molecular regulation of placental glucose transporters may be investigated in parallel with in vivo studies of transplacental glucose transfer and metabolism. When this thesis began, ovine specific cDNAs were not available for molecular studies of oGLUT1 and oGLUT3 gene expression.

Therefore, the objectives of this thesis were:

- Isolation of ovine GLUT1 and GLUT3 cDNAs to allow generation of ovine specific molecular probes.
- Investigation of oGLUT1 and oGLUT3 abundance and localisation in ovine placental tissue during normal placental development.
- Investigation of oGLUT1 and oGLUT3 regulation by factors known to regulate placental glucose transfer function and carbohydrate metabolism in late gestation ovine placenta.

**Generation of oGLUT1 and oGLUT3 cDNAs**

The isolation, cloning and sequence analysis of oGLUT1 (~1600 bp) and oGLUT3 (483 bp) cDNAs is described in Chapter 3, and sequence data arising from this thesis has been published in GenBank™/EMBL under accession numbers U89029 (oGLUT1) and U89030 (oGLUT3). The predicted 390 aa sequence obtained for oGLUT1 showed high homology with corresponding regions in bovine, human and rat (97 – 99% identity) deduced amino acid...
sequences. The same degree of sequence conservation was not apparent for oGLUT3. Although the oGLUT3 RT-PCR product amplified in this study was designed to incorporate a 161 aa fragment of GLUT3 with high cross-species homology, sequence identity with mouse and human GLUT3 deduced amino acid sequences was 84% and 88% respectively. This species specificity of sequence may play an important role in the regulation of oGLUT3, and merits further investigation.

GLUT1 and GLUT3 were targeted for study in ovine placenta because they predominate in rodent and human placenta (Hauguel-de Mouzon et al., 1997; Zhou and Bondy, 1993). However, other GLUT isoforms may be present in ovine placenta. Improved detection techniques have recently demonstrated the presence of GLUT2 and GLUT4 in human placenta (Quraishi and Illsley, 1999; Xing et al., 1998), and the unique nature of ruminant dietary physiology (Brockman, 1993) indicates that alternative GLUT isoforms may play important roles in ovine placental hexose transport. For this reason, any future cloning studies should focus on screening ovine placenta for other oGLUT isoforms.

Developmental regulation of placental oGLUT1 and oGLUT3 expression

The studies in Chapter 4 addressed the second objective for this thesis, and included analysis of oGLUT1 and oGLUT3 mRNA abundance (Northern) and localisation (in situ hybridisation), and oGLUT1 protein abundance (Western) and localisation (immunohistochemistry) in developing ovine placenta. Placentomes were sampled at d 45, 60, 90, 120, and 138 (term d 145 ±2).

Northern analysis demonstrated oGLUT1 and oGLUT3 have different temporal patterns of developmental regulation. Ovine GLUT1 mRNA abundance increased during early gestation, peaked at d 120, then decreased towards term. In contrast, oGLUT3 mRNA abundance continued to increase throughout gestation. These ontogenic changes in placental GLUT expression may reflect developmental changes occurring in the placenta. The pattern of change in oGLUT1 gene expression was similar to that seen for placental growth, whereas the pattern of increase in oGLUT3 gene expression paralleled the continuous maturation of placental transfer function that occurs during late gestation. These data support the hypothesis that oGLUT1 is important to placental glucose metabolism, and to maternal placental glucose transport, whereas oGLUT3 plays a prominent role in placental-fetal (or vice versa since glucose transport is bi-directional) glucose transport (Zhou and Bondy, 1993).
In contrast to oGLUT1 mRNA, Western analysis of oGLUT1 protein data showed that oGLUT1 protein abundance increased throughout gestation. These data indicate that oGLUT1 is regulated at the post-transcriptional level in late gestation ovine placenta. Post-transcriptional regulation of GLUT1 has previously been reported in rat brain (Boado and Partridge, 1993). Furthermore, post-transcriptional regulation of GLUT3 has been demonstrated in human placenta (Hauguel-de Mouzon et al., 1997). These reports together with the preliminary findings from the current study provide increasing evidence for multilevel regulation of placental GLUTs, and provide broad scope for future studies.

Developmental regulation of placental oGLUT1 and oGLUT3 localisation

In late gestation ovine placenta (d 126) oGLUT1 mRNA, though present in both maternal and fetal cells, was most strongly localised to maternal syncytiotrophoblast and villous tissue. Ovine GLUT3 was localised to the fetal side of the multinucleate syncytiotrophoblast.

Immunohistochemistry revealed that oGLUT1 protein localisation differed in early and late gestation. In early gestation (d 45), oGLUT1 was restricted to fetal trophoblast cells and the invading trophoblast cells of the terminal fetal villi. As gestation progressed oGLUT1 immunopositive staining increased in maternal villous tissue. By d 90 the maternal villi showed strong immunopositive staining for oGLUT1, and the intense immuno-signal in terminal fetal villi was lost. Such ontogenetic changes in oGLUT1 protein distribution may reflect placental growth and development. During early gestation the placenta undergoes rapid growth, and metabolically active mitotic trophoblast cells may be the primary glucose/energy consumers during rapid expansion of the fetal trophoblast. However, by d 90 placental DNA content is maximal (Ehrhardt and Bell, 1995), and maternal glucose may then be directed to placental metabolism and transport to the fetus.

These data provide further support for the hypothesis that, in late gestation, GLUT1 is primarily responsible for placental glucose uptake, whereas GLUT3 is responsible for transport between placenta and fetus (Zhou and Bondy, 1993). Limitations of in situ hybridisation and light microscopy made accurate cellular and sub-cellular localisation difficult, and with the benefit of experience it is clear that cellular localisation of the oGLUT signal could have been verified more easily by using immunohistochemical markers for structural orientation (eg, anti-cytokeratin to identify epithelial trophoblast cells). This approach should be adopted for future studies, providing suitable ovine specific antibodies against such markers are available.
Effects of maternal undernutrition or glycemia

Studies investigating the effects of maternal nutrient (glucose) supply on placental oGLUT expression demonstrated that placental oGLUT1 and oGLUT3 expression is regulated differently by acute versus long-term maternal glycemia. The current study provided no evidence for regulation of oGLUT gene expression by long-term (20 d) late gestation maternal undernutrition. However, analysis of individual placentomes revealed decreased average placentome weight and increased placentome number, indicating that changes may have occurred in placental morphology and metabolism to compensate for restricted maternal nutrition.

Insulin induced maternal hypoglycemia (modelling maternal undernutrition) resulted in increased oGLUT1 protein levels after short-term (48 h) maternal hypoglycemia, with a return to normal levels after long-term (7 – 10 d) maternal hypoglycemia. Northern analysis revealed no significant differences in oGLUT1 or oGLUT3 mRNA abundance when compared with control values. However, a significant decrease in oGLUT1 and oGLUT3 mRNA abundance between short-term (24 – 48 h) and chronic (34 – 36 d) maternal hypoglycemia samples indicated initial elevation and subsequent suppression of oGLUT1 and oGLUT3 gene expression during maternal hypoglycemia. Samples from animals subjected to acute (3 – 8 h) maternal hypoglycemia were unavailable for study. However, the elevated oGLUT mRNA abundance after short-term (24 – 48 h) maternal hypoglycemia indicated that acute regulation of placental oGLUT genes may be important, and future work is required to investigate placental oGLUT expression during the first 24 h of maternal hypoglycemia.

Glucose induced maternal hyperglycemia (modelling maternal diabetes) resulted in a 2.5-fold increase in oGLUT1, and a more than fourfold increase in oGLUT3 mRNA abundance after 3 – 8 h. By 24 – 48 h, expression of both genes had returned to normal and showed no further change with long-term maternal hyperglycemia. Conversely, Western analysis demonstrated no change in oGLUT1 protein levels after acute or short-term maternal hyperglycemia, but oGLUT1 protein levels increased after long-term (8 – 9 d) maternal hyperglycemia. The disparity between mRNA and protein data implies a biphasic response to acute and long-term maternal hyperglycemia, and provides further evidence for post-transcriptional regulation of placental oGLUT1.
Effects of fetal administration of GH or IGF-1

The current study found no evidence for regulation of oGLUT1 or oGLUT3 gene expression by long-term administration of bGH to the late gestation ovine fetus. However, this study was far from definitive. Future studies should include investigation of placental oGLUT mRNA and protein expression after acute fetal GH infusion, and include measurements of placental glucose uptake, transfer and metabolism.

Initial observations indicated that long-term administration of IGF-1 to the late gestation ovine fetus resulted in significant decreases in average placentome weight, and in oGLUT1 and oGLUT3 mRNA abundance. However, comparison with data from the original experiment (Lok et al., 1996) suggests placentome weight data from the current study was not consistent with placentome weight data as a whole. Furthermore, previous studies have shown IGF-1 promotes placental maturation and hastens development of placental transfer function (Owens, 1991). These observations taken together cast serious doubt on the oGLUT expression data from the current study, and any future study on the effects of fetal IGF-1 on placental oGLUT expression should re-investigate long-term infusion.

The final experiment described in this thesis investigated placental oGLUT gene expression in response to acute IGF-1 infusion in normal fetuses (N+IGF-1) and in fetuses that were growth restricted due to experimentally induced placental embolisation (E+IGF-1) (Jensen et al., 1999). In the current study, the effects of IGF-1 infusion could not be separated from the effects of placental embolisation because RNA from control animals (saline infusion only) was degraded. Analysis of Northern data from the two remaining experimental groups showed no significant difference in placental oGLUT1 mRNA abundance between N+IGF-1 and E+IGF-1 treated fetuses, whereas abundance of placental oGLUT3 mRNA was significantly greater in E+IGF-1 than N+IGF-1 fetuses. Jensen et al (1999) demonstrated that embolised placentae in growth restricted fetuses (E+IGF-1) were resistance to the metabolic effects of acute IGF-1 infusion. Therefore, it is unlikely that the increase in oGLUT3 mRNA observed in the current study in E+IGF-1 fetuses was due to acute IGF-1 infusion. Two other interpretations are possible. The first is that placental oGLUT3 mRNA may have been up-regulated in E+IGF-1 animals by hypoxia or stress related factors (eg, cortisol) as a result of placental embolisation. The second interpretation is that oGLUT3 mRNA was down-regulated in normal animals by acute infusion of IGF-1 to the fetus. If so, regulation was unlikely to be direct because IGF-1 is known to up-regulate oGLUT3 (Kniss et al., 1994). Intermediary metabolites including lactate have been shown to suppress glucose transport.
(Fischer et al., 1997), and Jensen et al (1999) reported that acute IGF-1 infusion to normal, but not embolised, fetuses increased fetal blood lactate concentrations by approximately 25%. Taken together, these observations make lactate a strong candidate for regulation of placental oGLUT3 gene expression. These data recommend further studies into the role that intermediary metabolites (including lactate) play in ovine placental GLUT regulation.

7.1.1 Experimental model

Some (Hahn et al., 1998a) argue that animal models cannot be used for the study of transplacental glucose transport because the underlying mechanisms differ from those in humans. In humans, for example, GLUT3 protein is restricted to fetal endothelium (Hauguel-de Mouzon et al., 1997) and GLUT1 is the major placental glucose transporter. In contrast, GLUT3 plays a prominent role in rodent placental glucose transport (Boileau et al., 1995; Devaskar et al., 1994; Zhou and Bondy, 1993).

Sheep may not be an ideal model with which to study human placental glucose transport. Humans and sheep have very different placental morphology and dietary physiology. Furthermore, unlike standard laboratory animals (eg, rats), sheep may vary greatly in phenotype and genotype. An additional consideration is that large animal studies are relatively expensive and it is difficult to get sufficient numbers for powerful statistics. However, there are major advantages to recommend sheep. Mother and fetus can be catheterised to allow physiological studies on both sides of the placenta, and easy husbandry allows long-term experiments to be undertaken in conscious animals. Thus, molecular physiology may be investigated in tandem with in vivo metabolic and endocrine studies. For these reasons, future studies investigating placental glucose transport in sheep are valuable.

7.2 FUTURE WORK

Ovine placentome weight and morphology may be affected by a number of factors including gestational age and uterine position (Ehrhardt and Bell, 1997; Stevens et al., 1981). In the current study, efforts were aimed at maintaining consistency in placentome sampling. Unless otherwise stated, placentomes were late gestation (d 125 – d 143), type A (most abundant morphological type in local animals – see section 1.2.1.2 for definition of classification), and of similar size (~ 6g) and position in the uterus (midline, main body of uterus). Studies in this thesis examined the affects of gestational age on GLUT1 and GLUT3 gene expression (Chapter 4). However, for a more complete picture of ovine placental GLUT expression,
future studies should include representative samples of placentomes from different morphological types (inverted type A to everted type D), various sizes, and uterine positions (eg, uterine horn versus main body of the uterus).

A number of avenues for further research have been mentioned in the preceding summary. However, two in particular are pressing. A major limitation to this thesis was the lack of oGLUT3 protein data. Although the abundance of oGLUT3 mRNA in ovine placenta suggests it is important to ovine placental glucose transport, lessons learnt from human GLUT3 regulation (Hauguel-de Mouzon et al., 1997) have emphasised that it is not possible to extrapolate from mRNA to protein expression. Antibodies to oGLUT3 are now available (Ehrhardt and Bell, 1997), and investigation of oGLUT3 protein expression during placental development and function are of highest priority.

The studies in this thesis have demonstrated that placental oGLUTs may be regulated at the molecular level by maternal and fetal factors. However, further studies are required to isolate the individual regulatory factors and to study the regulatory mechanisms. The current study and others have indicated that glucocorticoids (Hahn et al., 1999), insulin and IGF-1 (Gordon et al., 1995; Kniss et al., 1994) may play important roles in regulation of placental glucose transport, and fetal infusion studies using these hormones may answer some of the questions raised by this thesis. In addition, it is increasingly clear that GLUTs are subject to multiple levels of regulation, and that this allows GLUTs to respond extremely rapidly to changes in glucose homeostasis. In the current study, Northern and Western analysis measured ‘snap shots’ of oGLUT1 and oGLUT3 mRNA and oGLUT1 protein abundance. However, alterations in mRNA stability may not cause any observable change in mRNA abundance, and relatively small changes at each level of regulation may result in large change in glucose transport. Future studies investigating placental oGLUT regulation should focus either on examining the different levels of oGLUT regulation by specific factors (using in vivo techniques and trophoblast cell culture), OR on regulation of placental oGLUT expression during physiological changes in mother and fetus by measuring activated (plasma membrane associated) protein expression. The critical importance of glucose to metabolism suggests that acute regulation of placental oGLUT expression is an important area for future research. In addition, substrate interactions within the conceptus indicate that fetal regulation of placental oGLUT3 is of paramount importance to partitioning of glucose between placenta and fetus, and investigation of oGLUT3 regulation should therefore be a priority.
APPENDICES

APPENDIX 1: cDNA Library screening and recombinant DNA techniques

1.1 λ diluent
Tris.HCl (pH 7.5) 10 mM
MgSO₄.7H₂O 10 mM

1.2 Denaturing solution
NaCl 1.5 M
NaOH 0.5 M

1.3 Neutralising solution
NaCl 1.5 M
Tris.HCl (pH 7.2) 0.5 M
EDTA 0.001 M

1.4 Prehybridisation buffer (screening library)
SSPE 5 x
Denhardts 5 x
SDS 0.5%
Add 100 μg/ml of denatured salmon sperm DNA (ss DNA). Make up to 25 ml with sterile H₂O. Use 3 ml buffer per membrane.

1.5 20 x SSPE (saline sodium phosphate EDTA)
NaCl 3.6 M
Sodium phosphate 0.2 M
EDTA 0.02 M
pH 7.7
1.6 20 x SSC (standard saline citrate)

NaCl  
3 M

Na₃Citrate.2H₂O  
0.3 M

Adjust pH to 7.0 with 1 M HCl.

1.7 SM (storage media)

NaCl  
5.8 g

MgSO₄.7H₂O  
2 g

1 M Tris.HCl (pH 7.5)  
50 ml

2% gelatin  
5 ml

ddH₂O to 1 litre.

1.8 LB (Luria Bertani media)

Bacto-tryptone (Casein hydrolysate)  
10 g

Bacto-yeast extract  
5 g

NaCl  
10 g

Glucose (omit if using blue/white selection)  
1 g

Adjust to pH 7.5 and add dd H₂O to 1 litre.

To make LB agar plates add 15 g agar to 1 litre LB broth and autoclave.

1.9 Terrific Broth

Make 900 ml base broth and autoclave:

Bacto-tryptone  
12 g

Bacto-yeast extract  
24 g

Glycerol  
4 ml (5 g)

Add filter sterilised:

0.17 M KH₂PO₄  
100 µl

0.72 M K₂HPO₄  
100 µl
1.10 SOB media

Bacto-tryptone 20 g
Bacto-yeast extract 5 g
5 M NaCl 2 ml
2 M KCl 1.25 ml
H₂O to 1 litre.

Make a 2 M stock of Mg⁺⁺ using 1 M MgCl₂·6H₂O plus 1 M MgSO₄·7H₂O. Sterile filter (0.2 µm). Just prior to use add 20 mM Mg⁺⁺ to media.

1.11 FRM media

NaCl 5 g
MgCl₂ 2 g
NZ Amine 10 g
Yeast extract 5 g
Casamino Acids 2 g
Maltose 2 g
pH 7.5 and H₂O to 1 litre.

For bottom agar add 15 g of bacto-agar/litre of FRM.
For top agarose add 7.5 g of agarose/litre FRM.
Always use agarose for phages.

1.12 TE (Tris EDTA)

Tris.HCl (pH 7.4, 7.5, 8.0) 10 mM
EDTA (pH 8.0) 1 mM

1.13 GTE (glucose Tris EDTA)

Glucose 50 mM
Tris (pH 8.0) 25 mM
EDTA (pH 8.0) 10 mM
Store at 4°C.

1.14 NaOH/SDS

NaOH 0.2 M
SDS 1%
Prepare fresh.

1.15 KAc (potassium acetate)

5 M KAc 60 ml
Glacial acetic acid 11.5 ml
dd H₂O to 100 ml. Store at 4°C.

1.16 20% Bluogal

Bluogal 100 μg
Make up in 500μl dimethylformamide.
APPENDIX 2: NORTHERN ANALYSIS

2.1 6 x RNA dye

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
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<tr>
<td>DNA dye</td>
<td>1 x</td>
</tr>
<tr>
<td>Deionized formamide</td>
<td>50% (v/v)</td>
</tr>
<tr>
<td>MOPS</td>
<td>20 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>6.5% (v/v)</td>
</tr>
</tbody>
</table>

2.2 Methylene blue stain

<table>
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<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>0.04 g (0.02% w/v)</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>13.6 g (0.5 M)</td>
</tr>
</tbody>
</table>

Adjust pH 5.2 using glacial acetic acid.

DEPC H₂O to 200ml.

Wet membrane in 2 x SSC
Replace with methylene blue and stain for 10 min.
Destain with 1x SSC 10 min.
Scan membrane.

Remove stain before hybridisation by incubating in 0.2 x SSC/1% SDS for 15 min at RT.
Change solution as it becomes coloured.

2.3 10 x MOPS

<table>
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<th>Concentration</th>
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<tr>
<td>MOPS</td>
<td>0.2 M</td>
</tr>
<tr>
<td>NaOAc</td>
<td>0.05 M</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>0.01 M</td>
</tr>
<tr>
<td>pH 7 (NaOH)</td>
<td></td>
</tr>
<tr>
<td>ddH₂O to 1 litre</td>
<td></td>
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2.4 Northern hybridisation buffer

<table>
<thead>
<tr>
<th>Component</th>
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<tr>
<td>Deionized formamide</td>
<td>50%</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
</tr>
<tr>
<td>NaCl</td>
<td>1 M</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>10%</td>
</tr>
<tr>
<td>Denatured ss DNA</td>
<td>100 μg/ml.</td>
</tr>
</tbody>
</table>
APPENDIX 3: IN SITU HYBRIDISATION

3.1 Oligonucleotide probe hybridisation buffer

20 x SSC
Dextran sulfate
Deionized formamide
Heat to ~50°C and vortex 30 min. Then add:
Poly A⁺
ss DNA
tRNA
1 M DTT
50 x Denhardt’s solution

0.25 mg/ml
0.25 mg/ml
0.25 mg/ml
100 mM
0.5 x

3.2 0.1 M Phosphate buffered saline (PBS)

Solution A: 0.2 M
NaH₂PO₄

24.0 g/litre

Solution B: 0.2 M
Na₂HPO₄

28.4 g/litre

0.1 M PB (pH 7.4)
Mix solution A:solution B (1:4)
To make 0.1 M PB dilute 1:1 with ddH₂O
(NB: If PBS required add 9 g of NaCl/litre).

4% paraformaldehyde in 0.1 M PB (or PBS) (pH 7.4)
Add 4 g paraformaldehyde to 100 ml 0.1 M PB (or PBS)
Add 1 – 2 drops 10 N NaOH and heat to 60°C stirring
Filter and cool.
APPENDIX 4: WESTERN ANALYSIS

4.1 Protein homogenisation buffer

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<th>Component</th>
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<tr>
<td>Sucrose</td>
<td>250 mM</td>
</tr>
<tr>
<td>HEPES (pH 7.4)</td>
<td>20 mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>2 mM</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>3 mM</td>
</tr>
<tr>
<td>PMSF (in 100% EtOH)</td>
<td>1 mM</td>
</tr>
<tr>
<td>NEM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Benzamidine hydrochloride</td>
<td>5 mM</td>
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4.2 4 x Protein sample loading buffer (reducing)

<table>
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<tbody>
<tr>
<td>Tris-HCl (0.5 M, pH 6.8)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>2-β-mercaptoethanol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.05% w/v</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4 ml</td>
</tr>
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</table>

4.3 Polyacrylamide/bis-acrylamide solution (4 minigels)

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>4% 7.5% 12%</td>
</tr>
<tr>
<td>Tris-HCl (1.5 M, pH 8.8)</td>
<td>6.4 ml 10.93 ml 8.7 ml</td>
</tr>
<tr>
<td>Tris-HCl (0.5 M, pH 6.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>100 μl 200 μl 200 μl</td>
</tr>
<tr>
<td>Acrylamide/N’N-Bis-acrylamide (40%)</td>
<td>0.98 ml 3.75 ml 6 ml</td>
</tr>
<tr>
<td>Ammonium persulphate (10%)</td>
<td>50 μl 100 μl 100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl 10 μl 10 μl</td>
</tr>
<tr>
<td>Total</td>
<td>10 ml 20 ml 20 ml</td>
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</table>
4.4 5 x Running gel buffer (pH 8.3)

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Tris</td>
<td>9 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>43.2 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>30 ml</td>
</tr>
<tr>
<td>ddH₂O to 600 ml</td>
<td></td>
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4.5 Towbins buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.12 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>57.6 g</td>
</tr>
<tr>
<td>AR Methanol</td>
<td>800 ml</td>
</tr>
<tr>
<td>ddH₂O to 4 litres and store at 4°C.</td>
<td></td>
</tr>
<tr>
<td>Re-use once only.</td>
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4.6 Membrane stripping buffer

<table>
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<tr>
<td>β-mercaptoethanol</td>
<td>100 mM</td>
</tr>
<tr>
<td>10% SDS</td>
<td>20 ml (2%)</td>
</tr>
<tr>
<td>1 Tris-HCl (pH 6.7)</td>
<td>6.25 ml</td>
</tr>
</tbody>
</table>

Submerge membranes in stripping buffer and incubate at 50°C for 30 min.
Appendices

APPENDIX 5: IMMUNOHISTOCHEMISTRY

5.1 Fixation process for paraffin embedded tissues

Fix tissue in freshly made 4% paraformaldehyde/0.1 M PBS at 4°C overnight. Then process as follows:

- 50% alcohol: 30 min
- 70% alcohol: 30 min
- 90% alcohol: 30 min
- 95% alcohol: 30 min
- 100% alcohol: 30 min
- 100% alcohol: 30 min
- 100% alcohol: 30 min
- Xylene: 30 min
- Xylene: 30 min
- Wax: 2 h
- Wax: 2 h
- Wax: 2 h

5.2 0.01 M PBS (pH 7.4)

- NaCl: 8 g
- Na₂HPO₄: 1.15 g
- KH₂PO₄: 0.2 g
- KCl: 0.2 g

ddH₂O to 1 litre.
APPENDIX 6: PUBLICATIONS ARISING FROM THIS THESIS


GenBank™/EMBL PUBLICATIONS

2. Ovine GLUT1

<table>
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<tr>
<th>LOCUS</th>
<th>OAU89029</th>
<th>1600 bp mRNA</th>
<th>MAM</th>
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<td>DEFINITION</td>
<td>Ovis aries glucose transporter type 1 (GLUT-1) mRNA, partial cDNA.</td>
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<tr>
<td>ORGANISM</td>
<td>Ovis aries</td>
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<td>Eukaryote; mitochondrial eukaryotes; Metazoa; Chordata; Vertebrata; Eutheria; Artiodactyla; Ruminantia; Pecora; Bovoida; Bovidae; Caprinae; Ovis.</td>
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<td>REFERENCE</td>
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<tr>
<td>AUTHORS</td>
<td>Currie,M.J., Bassett,N.S. and Gluckman,P.D.</td>
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<tr>
<td>TITLE</td>
<td>Ovine glucose transporter-1 and -3: cDNA partial sequences and developmental gene expression in the placenta</td>
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<tr>
<td>JOURNAL</td>
<td>Placenta (1997), 18, 393-401.</td>
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<tr>
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REFERENCE 1 (bases 1 to 483)
AUTHORS Currie, M.J., Bassett, N.S. and Gluckman, P.D.
TITLE Ovine glucose transporter-1 and -3: cDNA partial sequences and developmental gene expression in the placenta
JOURNAL Placenta (1997), 18, 393-401.

REFERENCE 2 (bases 1 to 483)
AUTHORS Currie, M.J., Bassett, N.S. and Gluckman, P.D.
TITLE Direct Submission
JOURNAL Submitted (07-FEB-1997) Research Centre for Developmental Medicine and Biology, School of Medicine, The University of Auckland, Auckland, Private Bag 92019, New Zealand.

FEATURES

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REFERENCES


References


References


References


Mellor, D. J. and Matheson, I. C. (1979). Daily changes in the curved crown-rump length of individual sheep fetuses during the last 60 days of pregnancy and effects of different levels of


References


Unterman, T., Lascon, R., Gotway, M. B., Oehler, D., Gounis, A., Simmons, R. A. and Ogata, E. S. (1990). Circulating levels of insulin-like growth factor binding protein-1 (IGFBP-1) and hepatic mRNA are increased in the small for gestational age (SGA) fetal rat


