The Role of Thyroid Hormones in Placental Development and

the Importance of the Thyroid Hormone Transporter MCT8

by

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Abstract

Thyroid hormones (THs) are important for fetoplacental development. Human intrauterine growth restriction (IUGR) is associated with malplacentation and reduced fetal circulating concentration of THs. The expression of the plasma membrane TH transporters MCT8, MCT10, LAT1, LAT2, OATP1A2 and OATP4A1 was characterised in human placental biopsies across gestation. The protein expression of MCT8 was increased in samples from severe IUGR compared with normal pregnancies. *In vitro*, tri-iodothyronine (T_3) decreased survival and increased apoptosis of IUGR compared with normal cytotrophoblasts, which was associated with increased MCT8 expression. In normal cytotrophoblasts, MCT8 upregulation decreased survival, whilst MCT8 silencing increased survival independently of T_3. In the extravillous trophoblast-like cell line, SGHPL-4, T_3 resulted in a significant increase in cell invasion when MCT8 was upregulated. Contrary to cytotrophoblasts, silencing MCT8 decreased apoptosis in SGHPL-4s independently of T_3. In mice, fetal to placental weight ratio was decreased in male MCT8-null compared with wild-type embryos. These findings support the hypothesis that THs have an important role in fetoplacental development and that IUGR is associated with changes in TH transport and responsiveness of the placenta. Furthermore, they highlight the importance of MCT8, which impacts on placental cells via both T_3-dependent and independent mechanisms.

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°C	Degrees Celsius
18S	Small subunit ribosomal RNA
Ab	Antibody
AMV	Avian myeloblastosis virus
APS	Ammonium persulphate
bp	Base pairs
BP	Blocking peptide
BrAcT$_3$	N-bromoacetyl-T$_3$
BSA	Bovine serum albumin
BSP	Bromosulphophthalein
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary
CNS	Central nervous system
cpm	Counts per minute
CRYM	μ-Crystallin
Ct	Cycle threshold value
CT(s)	Cytotrophoblast(s)
Cx	Connexin
D1	Deiodinase type I
D2	Deiodinase type II
D3	Deiodinase type III
DIT	Diiodinated tyrosine residue
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP(s)	Deoxynucleotide triphosphate(s)
DTT	Dithiothreitol
E14.5	Gestational day 14
E18.5	Gestational day 18
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ES	Embryonic stem cells
EVT(s)	Extravillous trophoblast(s)
FCS	Fetal calf serum
FLIP	Flice-like inhibiting protein

g	Gram
GFP	Green fluorescent protein
HAT	Heterodimeric amino acid transporter
HBSS	Hepes-buffered salt solution
hCG	Human chorionic gonadotrophin
HLA	Human leukocyte antigen
hPL	Human placental lactogen
HPT	Hypothalamus-pituitary-thyroid
HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IRD	Inner ring deiodination
IUGR	Intrauterine growth restriction
Jz	Junctional zone
Ka	Association constant
kDa	Kilodalton
L	Litre
LAT	L-Type amino acid transporter
LDH	Lactate dehydrogenase
Lz	Labyrinth zone
M	Molar
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
ME1	Malic enzyme 1
mg	Milligram
MHC	Major histocompatibility complex
MIT	Monoiodinated tyrosine residue
ml	Millilitre
mlU	Milliunits
mM	Millimolar
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTT	(3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide)
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NIS	Na^+/I^- symporter
nM	Nanomolar
nm	Nanometres
nmole(s)	Nanomole(s)

NS	Not statistically significant
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP(s)	Organic anion transporting polypeptide(s)
ORD	Outer ring deiodination
PAT(s)	Proton-assisted amino acid transporter(s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Bayley Psychomotor Developmental Index
PEST	Proline, glutamic acid, serine and threonine repeats
PGH	Placental growth hormone
pmole(s)	Picomole(s)
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
Rnasin	Ribonuclease inhibitor
rpm	Revolutions per minute
rT3	3,3,5'-tri-iodothyronine
RXR	Retinoic acid X receptor
SDHA	Succinate dehydrogenase complex, subunit A
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFCS	Charcoal-stripped fetal calf serum
SFM	Serum-free media
siRNA	Small interfering ribonucleic acid
SNAT(s)	Sodium-coupled neutral amino acid transporter(s)
SRY	Sex-determining region of chromosome Y
ST(s)	Syncytiotrophoblast(s)
SULT(s)	Sulphotransferase(s)
T_2	Di-iodothyronine
T_3	3,5,3'-tri-iodothyronine
T_4	Thyroxine
TAT-1	T-Type amino acid transporter
TBP	TATA box-binding protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tg	Thyroglobulin
TGF-β	Transforming growth factor-β
TH(s)	Thyroid hormone(s)
TMD(s)	Transmembrane domain(s)
TPO	Thyroid peroxidase

TR(s)	Thyroid hormone receptor(s)
TRE(s)	Thyroid hormone response element(s)
TRH	Thyrotropin releasing hormone
TRHR	Thyrotropin releasing hormone receptor
Triac	3,3',5-tri-iodothyroacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor
UGT	Diphosphate-glucuronosyltransferase
uNK(s)	Uterine natural killer cell(s)
v/v	Volume to volume ratio
VEGF	Vascular endothelial growth factor
VO	Vector only
vs	*Versus*
w/v	Weight to volume ratio
WB	Western immunoblotting
XIAP	X-linked inhibitors of apoptosis protein
XPCT	X-linked PEST containing transporter

Chapter 1: General Introduction

1.1   The human placenta

The placenta connects the developing fetus to the maternal circulation and facilitates the exchange of substances between the mother and the fetus. This includes the supply of oxygen and nutrients from the maternal circulation and the disposal of fetal waste. Fetal growth is thus dependent on transplacental transport. There are different placental types found in different mammalian species and they are classified according to the shape of the placenta and the number of layers that separate the maternal and the fetal circulations. In humans, the placenta is discoid and the placental villi are in direct contact with the maternal blood. The human placenta is therefore classified as discoid, haemochorial placenta. The human placenta is attached to the maternal uterine wall and in particular to the endometrium, the lining of the uterus that is transformed into the decidua following changes occurring during the menstrual cycle and with pregnancy.

1.1.1   Human placental development

1.1.1.1   Trophoblast differentiation and function

Fertilisation of the mature oocyte occurs in the ampulla region of the oviduct. Following fertilisation, the oocyte undergoes cleavage and compaction, whilst it is travelling towards the uterus. This leads to the formation of the morula, which consists of a group of internal and a group of external cells. The internal cells generate the inner cell mass, which will give rise to the embryo. The external cells give rise to the trophectoderm that will differentiate into trophoblast cells that form the placenta. The formation of the trophectoderm is the first differentiation event in mammalian development. The morula undergoes a process termed cavitation during which the trophoblast cells secrete fluid into

the morula leading to the formation of a blastocoel. The blastocoel expands and the inner cell mass is positioned on one side leading to the formation of the blastocyst. Once the blastocyst reached the uterus, it attaches to the maternal endometrium (Gilbert, 2006). The implantation of the blastocyst into the maternal endometrium is the result of interactions between the maternal cells of the endometrium and the trophoblast cells and can only occur in a restricted period of time that in humans is believed to span days 20-24 of the menstrual cycle (Lunghi *et al.*, 2007).

The human placenta consists of anchoring chorionic villi that are attached to the uterus and floating chorionic villi (**Figure 1-1**). Cytotrophoblast cells (CTs) are the proliferative cell type of the placenta that gives rise to other placental cell types (Lunghi *et al.*, 2007). In floating villi, some CTs differentiate to post-mitotic cells that fuse together to form a multinucleated syncytiotrophoblast (ST) layer (Huppertz and Kingdom, 2004). Following blastocyst implantation, mononuclear CTs break through the ST layer and they form chorionic villi that are attached to the maternal uterine wall (Huppertz and Peeters, 2005). At these sites, CTs aggregate forming CT columns and some differentiate into extravillous trophoblast cells (EVTs), post-proliferative cells with an invasive phenotype. EVTs invade the maternal endometrium and they can follow either the interstitial or the endovascular invasion route. Interstitial invasion involves the migration of EVTs through the decidua and the endometrium. Scattered giant cells that appear to form from the fusion of extravillous trophoblasts are found in the decidua and in the myometrium throughout gestation. Some interstitial EVTs reach the walls of the maternal spiral arteries and destroy the smooth muscle cells that are replaced by fibrinoid material. Endovascular EVTs invade the lumen of the arteries where they initially form arterial plugs and they prevent maternal blood from reaching the intervillous space until post-

menstrual week 12 (Huppertz and Peeters, 2005, Malassine *et al.*, 2003). Therefore, the early stages of fetal and placental development occur in a low oxygen environment. After week 12, endovascular EVTs migrate to the vessel walls where they replace the endothelial cells and thus, maternal spiral arteries are transformed to dilated, low-resistance vessels that facilitate unobstructed blood flow from the maternal circulation to the placenta (Craven *et al.*, 2000, Pijnenborg *et al.*, 1983).

The ST layer of the chorionic villi is the physical barrier that controls the uptake of substances from the maternal circulation and their efflux to the stromal core where they can reach the fetal capillaries (Jurisicova *et al.*, 2005). Small lipophilic substances can diffuse through the ST plasma membrane and cross the ST layer down their concentration gradient, however the transport of larger or hydrophilic molecules such as amino acids needs to be facilitated by transporter proteins at the apical and basal side of the ST. In addition, the ST has an endocrine function and is responsible for the synthesis of hormones and growth factors and their secretion to the maternal and/or the fetal circulation. The factors that are secreted by the ST include human chorionic gonadotrophin (hCG), human placental lactogen (hPL), placental growth hormone (PGH), oestrogen and progesterone and they are important for both the adaptation of the maternal metabolism to pregnancy and for fetal growth (Malassine *et al.*, 2003). The ST layer is continuously renewed as adjacent CTs fuse with the ST and aged ST nuclei and small amounts of cytoplasm are packed into syncytial knots, vesicles surrounded by membrane that are released into the maternal circulation (Huppertz *et al.*, 2006, Huppertz and Kingdom, 2004, Jurisicova *et al.*, 2005).
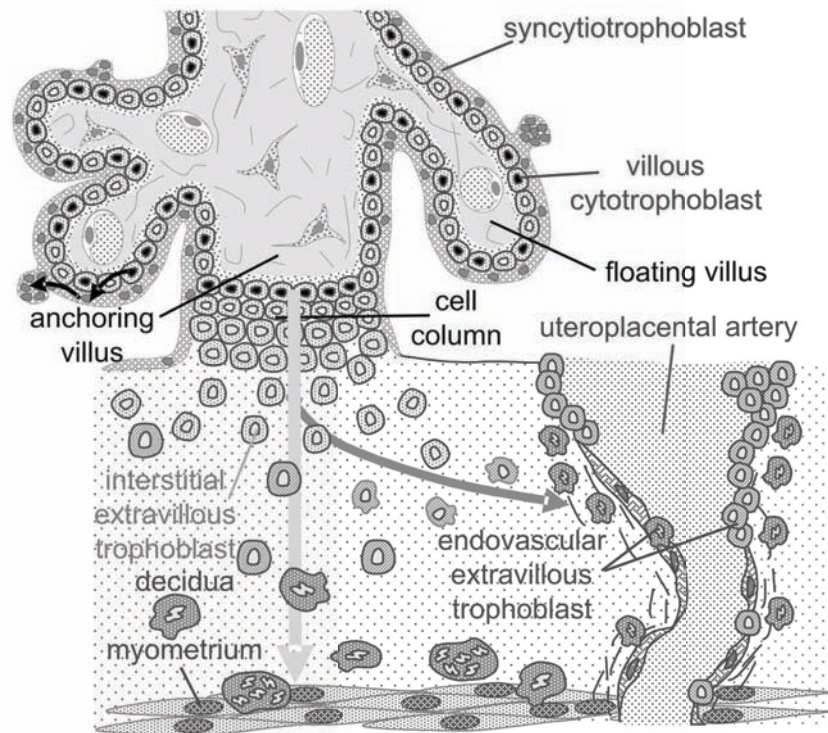
Figure 1-1: *Trophoblast differentiation pathways. In floating villi cytotrophoblasts fuse with the syncytiotrophoblast layer and aged syncytiotrophoblast nuclei are extruded into the maternal circulation as syncytial knots. In anchoring villi proliferating cytotrophoblasts form cell columns and some of them differentiate to extravillous trophoblasts that migrate to the maternal decidua. Extravillous trophoblasts follow one of two differentiation routes. Interstitial extravillous trophoblasts invade the maternal decidua and endometrium whilst endovascular extravillous trophoblasts invade the maternal spiral arteries. Figure from (Huppertz et al., 2006).*

1.1.1.2   Regulation of ST formation

The human ST regulates both transplacental transport and the secretion of hormones and growth factors into the maternal and fetal circulations. Trophoblast turnover from the proliferative CT cells to the post-mitotic ST cell layer and extrusion of aged ST nuclei to the maternal circulation as syncytial knots ensures the renewal of the ST layer. The fusion of CTs to the ST layer involves the initial stages of the apoptotic

cascade, whilst the extrusion of aged nuclei to the maternal circulation involves the execution stages of the apoptotic cascade (Huppertz and Kingdom, 2004). It has been shown that the initiator caspase 8 is active in CTs (Huppertz *et al.*, 1999) and is required for cell fusion, since blockage of caspase 8 expression or activity reduced trophoblast fusion in villous explant cultures (Black *et al.*, 2004). On the other hand, the effector caspases 3 and 6 that are part of the final execution stages of apoptosis are not active in CTs (Huppertz *et al.*, 1999). Initiator caspases degrade cytoskeletal proteins, such as fodrin, and they are involved in the externalisation of phosphatidylserine, a negatively charged amino phospholipid (Huppertz and Kingdom, 2004). The degradation of cytoskeletal elements and the re-arrangement of charges in the plasma membrane facilitate the re-organisation of the cell membrane that is necessary for cell fusion (Huppertz and Kingdom, 2004). Effector caspase activity is detected in specific sites of the ST layer at tips of villi, where aged, apoptotic nuclei accumulate and are packed in syncytial knots and extruded into the maternal circulation. The packaging of this apoptotic material in membrane-sealed structures ensures that there is no inflammatory response when it is released into the maternal circulation and the syncytial knots are engulfed by macrophages in the capillary bed of the maternal lung (Huppertz and Kingdom, 2004). The mechanisms that block the transition from the initiator to the execution stages of apoptosis in the differentiating CT and restrict its activation to defined areas within the ST are not completely understood. It is thought that anti-apoptotic molecules, such as members of the Bcl-2 family, actin-regulatory proteins, such as gelsolin and inhibitory proteins, such as the X-linked inhibitors of apoptosis protein (XIAP) and flice-like inhibiting protein (FLIP) contribute to the regulation of the final stages of apoptosis via inhibiting the activity of effector and/or initiator caspases (Huppertz and Kingdom, 2004).

The regulation of the apoptotic pathways within trophoblast cells is thus tightly linked to the formation and maintenance of the ST layer.

In addition to the apoptotic cascade, more pathways are thought to be involved in the differentiation of the proliferating CT leading to its fusion with the ST layer and research is currently ongoing to better define the mechanisms that are regulating this differentiation process. It has been shown that oxygen levels and the concentration of molecules, such as cyclic adenosine monophosphate (cAMP) and vascular endothelial growth factor (VEGF), regulate the expression levels of different transcription factors that have been associated with the fate of CT cells by either blocking mitosis and promoting cell fusion (GCM1, AP-2 and Sp) or maintaining proliferation and inhibiting fusion (Hash-2 and HIF-1) (Lunghi *et al.*, 2007). Cell-cell communication facilitates the diffusion of such molecules and it has been shown that connexins (Cx), gap junction proteins that form transmembrane channels and facilitate intercellular communication by allowing the diffusion of ions and small molecules such as cAMP, cyclic guanosine monophosphate (cGMP), inositol triphosphate and calcium ions, play a role in the regulation of trophoblast differentiation (Malassine and Cronier, 2005). Cx43 protein is localised between CTs and between STs and adjacent CTs indicating that it may be important for ST formation (Cronier *et al.*, 2002). Furthermore, *in vitro* studies have shown that blockage of Cx43 in primary cultures of trophoblast cells via treatment with heptanol, a non-specific junctional uncoupler that blocks all connexin channels (Cronier *et al.*, 2003), or via silencing of Cx43 expression using small interfering RNA (Frendo *et al.*, 2003) reduced the formation of syncytia, indicating that Cx43 is required for syncytialisation. There is also evidence that syncytins, envelope proteins derived from human endogenous retroviruses, are important in ST formation (Chen *et al.*, 2008a, Mi *et*

al., 2000, Muir *et al.*, 2004, Rote *et al.*, 2004). Silencing of syncytin-1 or syncytin-2 expression impaired the fusion of primary CTs *in vitro* (Vargas *et al.*, 2009). However, the pathways that are involved in the regulation of CT fusion by syncytins are not well understood yet. In addition, evidence has emerged that CD98, a membrane protein that dimerises with the light chains of amino acid transporters thus forming functional amino acid transporters, has also a role in cell fusion. In experiments with the choriocarcinoma cell line, BeWo, the protein expression of CD98 increased following stimulation of cell fusion by treatment with forskolin (Kudo and Boyd, 2004). Furthermore, knockdown of CD98 expression decreased both the number of fused BeWo cells and hCG secretion, thus suggesting a role for CD98 in trophoblast fusion (Kudo and Boyd, 2004). The mechanisms that mediate the role of CD98 in the fusion of BeWo cells are not completely understood. CD98 has multiple glycosylation sites and glycosylation appears to be necessary for CD98-induced cell fusion (Dalton *et al.*, 2007). Furthermore, CD98 is associated with galectin 3, a receptor for ligands such as lactose, as shown by co-immunoprecipitation assays and by co-localisation of CD98 and galectin 3 at the plasma membrane as well as in the cytoplasm and nucleus. The importance of the interaction between CD98 and galectin 3 for cell fusion is indicated by the decreased fusion of BeWo cells following disruption of galectin 3 and CD98 binding (Dalton *et al.*, 2007). As research on the mechanisms that are involved in CT fusion continues, more molecules that are involved in the differentiation pathways determining the fate of CTs are likely to be identified.

1.1.1.3   *Regulation of EVT invasion*

The differentiation of trophoblasts along the invasive phenotype and invasion into the maternal endometrium and transformation of the maternal spiral arteries is necessary

to facilitate the unobstructed flow of blood from the maternal circulation. Inadequate transformation of maternal spiral arteries has been associated with pregnancy complications such as intrauterine growth restriction (IUGR) and pre-eclampsia, whilst excessive EVT invasion is seen in molar pregnancies and choriocarcinomas (Lunghi *et al.*, 2007). Normal placentation is dependent upon optimal regulation of EVT invasion both spatially (decidua and the maternal endometrium, the inner third of myometrium) and temporally (early pregnancy) (**Figure 1-2**).

The initial stages of decidualisation of the maternal endometrium and transformation of the maternal spiral arteries are thought to occur in the late secretory phase of the menstrual cycle and are trophoblast-independent (Brosens *et al.*, 2002, Huppertz and Peeters, 2005, Lunghi *et al.*, 2007). These initial changes are regulated by the secretion of oestrogen and progesterone during the menstrual cycle that impact upon endometrial stromal cells and uterine immune cells, such as macrophages and uterine natural killer cells (uNK), resulting in altered steroid metabolism, remodelling of the extracellular matrix and cytoskeleton and altered expression of intracellular enzymes, growth factors, cytokines and their receptors (Brosens *et al.*, 2002). Intercellular signalling between smooth muscle cells and endothelial cells of the maternal spiral arteries result in the initial events of spiral artery remodelling that include intimal swelling, perturbation of arterial walls and disorganised vascular muscle cells and result to lumen dilation (Brosens *et al.*, 2002, Huppertz and Peeters, 2005). The remodelling of the maternal spiral arteries and the decidualisation of the myometrium are necessary for implantation and placentation and they regulate subsequent trophoblast invasion.

EVT invasion is required for further remodelling of maternal spiral arteries. Cells in CT columns that are in direct contact with the basal membrane of the villous maintain

their proliferative capacity whilst cells in distal areas of the CT columns differentiate to post-proliferative cells with an invasive phenotype (Huppertz and Peeters, 2005). It is thought that intercellular communication plays a role in determining differentiation of these cells and it has been found that proliferative cells in CT columns express the gap junctional protein Cx40 (Malassine and Cronier, 2005). Cx40 can not form functional channels with most other connexins, therefore it is hypothesised that Cx40-expressing cells in CT columns can not communicate with Cx43-expressing cells that differentiate into ST. EVTs that invade the maternal decidua have lost Cx40 expression, whilst EVT aggregates in the decidua co-express Cx40, Cx32 and Cx43. It is therefore hypothesised that Cx40 expression may promote cell proliferation, whilst loss of Cx40 expression may result in lack of cell communication and differentiation along the invasive pathway (Malassine and Cronier, 2005). Further research is necessary in order to identify the messenger molecules that are exchanged between Cx40-positive cells and favour cell proliferation.

Interstitial EVT invasion into the maternal myometrium is governed by interactions between EVTs and cells of the maternal decidua. Maternal cells secrete growth factors and cytokines that promote (e.g. VEGF) or inhibit (e.g. TGF-β) EVT invasion (Lunghi *et al.*, 2007). Furthermore, EVTs express cell surface molecules, such as the MHC class I molecules HLA-C, HLA-E and HLA-G that are ligands for killer immunoglobulin receptors present in uNK cells. Interactions between EVTs and uNKs regulate the expression of adhesion molecules, such as $\alpha 5\beta 1$ and $\alpha 1\beta 1$ integrins and VE cadherin, and the secretion of the degradative enzymes, matrix metalloproteinases, by EVTs, thus promoting EVT invasion (Lunghi *et al.*, 2007). Some interstitial EVTs migrate deep in the maternal endometrium and they eventually aggregate and fuse to form

giant cells. EVT fusion is promoted by TGF-β that is produced in the uterine decidua and such a mechanism might prevent excess invasion by interstitial EVTs (Brosens *et al.*, 2002). TGF-β is activated by plasmin, a protein that is generated by EVTs during invasion suggesting that there is the potential for autoregulation of interstitial EVT invasion (Brosens *et al.*, 2002).

Other EVTs follow the endovascular invasion route and invade the lumen of the spiral arteries where they initially create arterial plugs. After week 12 of pregnancy the plugs start to dislocate allowing blood flow from the maternal circulation and endovascular EVTs migrate in a retrograde direction within spiral arteries (Huppertz and Peeters, 2005, Pijnenborg *et al.*, 2006). The motility and migration of EVTs within spiral arteries is thought to be regulated by the increased oxygen level and the flow stress due to the blood stream that induce changes such as the expression of adhesion molecules like β1 integrin by endovascular EVTs (Pijnenborg *et al.*, 2006). Endovascular EVTs express hemoxygenase that facilitates the production of carbon monoxide, a vasodilator that causes further dilation of the arterial wall (Lunghi *et al.*, 2007). Furthermore, endovascular EVTs secrete matrix metalloproteinases that can degrade the subendothelial basement membranes of the vessel walls (Pijnenborg *et al.*, 2006). Spiral artery remodelling is completed by the reduction in the elastic fibres of the arterial walls and the replacement of smooth muscle cells by endovascular EVTs (Huppertz and Peeters, 2005). These changes result in a three-fold increase in the lumen diameter and reduced contractility of spiral arteries and facilitate constant unobstructed blood flow from the maternal circulation to the placental intervillous space (Huppertz and Peeters, 2005).

The differentiation of trophoblast cells along the invasive phenotype and their subdifferentiation towards the interstitial or the endovascular route is governed by

complex mechanisms that involve intercellular communication, growth factor and cytokine signalling and matrix metalloproteinase activity (Huppertz and Peeters, 2005, Lunghi *et al.*, 2007, Pijnenborg *et al.*, 2006). It is postulated that changes in oxygen level that are induced by changes in spiral arteries affect cell differentiation. Low oxygen tension is thought to promote cell proliferation whilst high oxygen tension favours differentiation towards the invasive phenotype (Pijnenborg *et al.*, 2006). Initial data suggest that apoptotic mechanisms may also play a role in the regulation of EVT invasion. EVTs that display characteristics of the late apoptotic changes were localised in the deeper part of the endometrium suggesting that interactions between maternal decidual cells and EVTs may regulate EVT apoptosis and thus contribute to the spatial regulation of EVT invasion to the decidua, the upper part of the myometrium (Huppertz and Peeters, 2005). Further research is likely to characterise EVT invasion in more detail and identify more pathways that participate in the regulation of EVT differentiation and mediate the interactions between maternal and fetal cells at the placental bed.
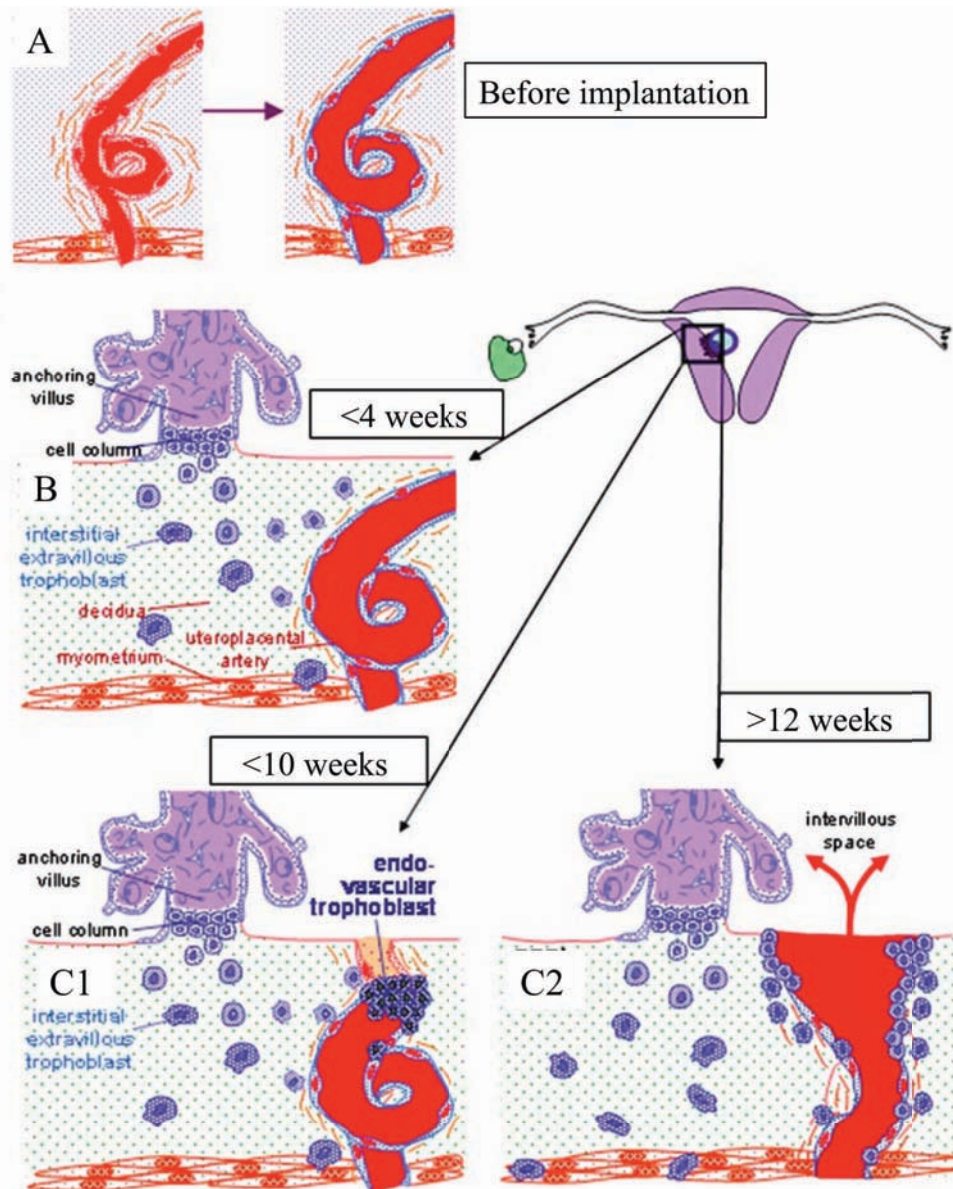
Figure 1-2: *The transformation of maternal spiral arteries during intrauterine pregnancy occurs in three phases. (A) The first phase is independent of trophoblast invasion. It involves the disorganisation of maternal vascular smooth muscle cells and the perturbation of arterial walls leading to lumen dilation and it is believed to be regulated by paracrine interactions between maternal smooth muscle cells and endothelial cells. (B) In the second phase, interstitial EVTs invade the maternal myometrium. (C) During the third phase, endovascular EVTs reach the spiral arteries where they initially create arterial plugs (C1). After week 12 of pregnancy arterial plugs are dislocated and EVTs replace the endothelial smooth muscle cells resulting in dilated non-contractile vessels and thus allowing unobstructed blood flow to the intervillous space (C2). Figure modified from (Huppertz and Peeters, 2005).*

1.1.2 Intrauterine growth restriction (IUGR)

IUGR is the failure to achieve the genetic growth potential of the fetus (Scifres and Nelson, 2009). IUGR has an impact on the function of various fetal organs including the cardiovascular system, lungs, pancreas, skeletal muscle and the brain. IUGR is a significant cause of perinatal mortality and morbidity (Sankaran and Kyle, 2009) with 10% of very low birthweight babies having some physical handicap (Gaffney *et al.*, 1994) and a further 5% demonstrating neurodevelopmental delay at the age of 9 years (Kok *et al.*, 1998). Furthermore, IUGR is associated with a life-long increased risk for hypertension, cardiovascular disorders and susceptibility to diabetes (Sankaran and Kyle, 2009, Scifres and Nelson, 2009). Estimated fetal weight less than the 10th percentile for gestational age in combination with increased head/abdomen circumference ratio, reduced amniotic fluid volume (oligohydramnios), vasodilation of the fetal middle cerebral artery circulation and increased impedence to pulsatile blood flow in the umbilical arteries are the criteria used for the diagnosis of IUGR (Chang *et al.*, 1993). The causes of IUGR are diverse and include aneuploidies, non-aneuploid genetic syndromes, infections, such as cytomegalovirus infection, metabolic factors and placental disorders (Scifres and Nelson, 2009). Uteroplacental insufficiency is associated with 80-90% of all IUGR cases (Sankaran and Kyle, 2009) as evidenced by the significantly smaller size of the IUGR placenta (Mayhew *et al.*, 2003). Other cases are associated with maternal calorie restriction, low protein diet or maternal diabetes (Sankaran and Kyle, 2009).

1.1.2.1 Impaired placental development in IUGR

Histological assessment of placental and placental bed sections from IUGR pregnancies has revealed changes in both spiral artery remodelling and placental

morphology in pregnancies complicated by IUGR. In normal pregnancies, maternal spiral

arteries in both the decidua and the endometrium are transformed by endovascular EVTs

leading to replacement of the contractile smooth muscle cells by endovascular EVTs and

to vasodilation. Maternal spiral artery remodelling is defective in IUGR (Pijnenborg *et al.*,

2006) particularly in the myometrial section (Burton *et al.*, 2009b) (**Figure 1-3**).
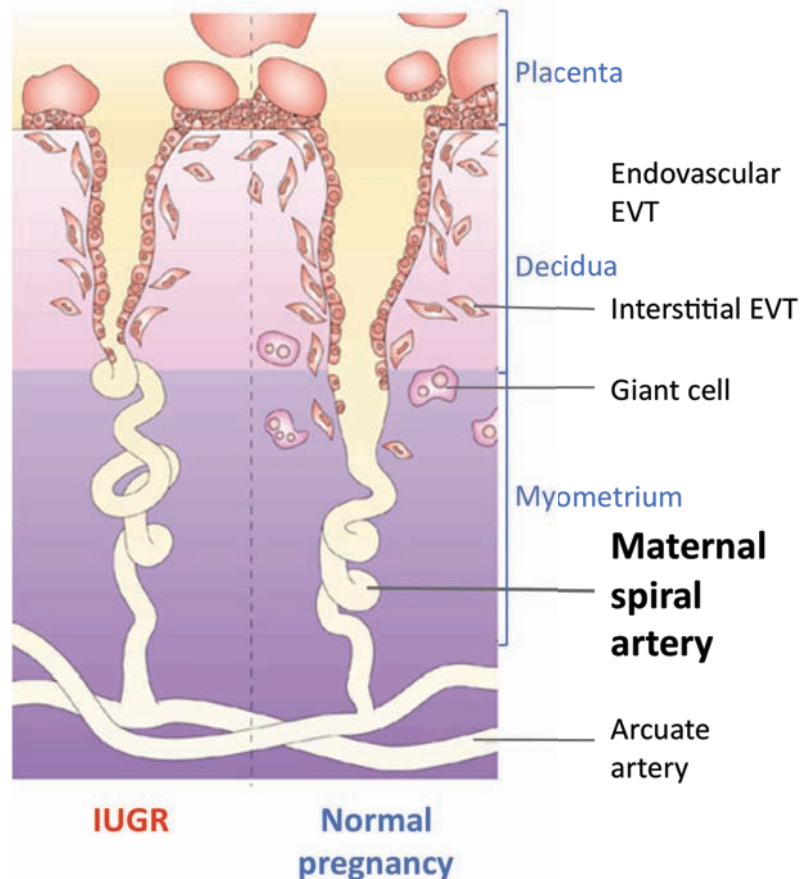
Figure 1-3: Intrauterine growth restriction is associated with inefficient transformation of the maternal spiral arteries resulting in a smaller lumen diameter of spiral arteries in IUGR compared with normal pregnancies. Figure modified from (Moffett-King, 2002).

Examination of spiral artery segments in the decidua and endometrium regions of

placental bed biopsies revealed that in sections from IUGR pregnancies 60% of the spiral

arteries were transformed in the decidua and only 10% were transformed in the

endometrium, in contrast to complete transformation of spiral arteries in both regions in normal pregnancy sections (Khong *et al.*, 1986). Examination of placental sections from normal and IUGR pregnancies revealed that in addition to the smaller placental size in IUGR pregnancies, the intervillous space volume was reduced and there was a decrease in villous tissue that equally affected trophoblast, stromal and capillary components of the villous structure and was accompanied by smaller exchange surface area (Mayhew *et al.*, 2003).

Although, IUGR is normally not manifested until the second half of pregnancy, it is thought that the placental maldevelopment that is associated with IUGR is established in early pregnancy when maternal spiral artery remodelling normally takes place. However, the factors that result in insufficient transformation of the maternal spiral arteries in IUGR are not completely understood. Insufficient invasion and transformation of the maternal spiral arteries in the first trimester may occur as a result of impaired decidualisation or impaired invasion by EVTs or as a combination of both. Decidual remodelling normally occurs during the menstrual cycle, and is priming the endometrium and the maternal spiral arteries for invasion by EVTs. In pregnancies complicated by IUGR there are changes in the recruitment of uNKs and the secretion of cytokines, such as IFN-γ, that impair the transformation of the maternal endometrium (Brosens *et al.*, 2002). Furthermore, increased recruitment of macrophages (Reister *et al.*, 1999, Reister *et al.*, 2001) and failure of maternal endothelial cells to express selectins (Burrows *et al.*, 1994) may contribute to the decreased spiral artery remodelling observed in IUGR pregnancies. Insufficient spiral artery remodelling in IUGR could also result from abnormalities in the differentiation of trophoblasts along the invasive phenotype. Even though, no changes have been observed in interstitial trophoblast invasion with IUGR

(Pijnenborg *et al.*, 1991), there is reduced endovascular invasion characterised by reduced number of EVTs in arterial walls and increased incidence of apoptosis in endovascular EVTs with IUGR (Kadyrov *et al.*, 2006). It is likely that the aforementioned changes in maternal cells contribute to the reduced endovascular invasion in IUGR pregnancies. Reduced expression of adhesion molecules, such as selectins, by maternal endothelial cells is likely to impair the ability of endovascular EVTs to migrate within the lumen of the spiral arteries. Furthermore, changes in immune responses of maternal cells may induce apoptosis of endovascular EVTs as evidenced by the ability of activated macrophages to induce apoptosis of EVT-like cells *in vitro* (Reister *et al.*, 2001).

Insufficient endovascular invasion results in high resistance spiral arteries that retain their contractility. Initially it was thought that impaired spiral artery remodelling would alter blood flow resulting in placental hypoxia or hyperoxia (Huppertz and Peeters, 2005). However, it is now thought that impaired spiral artery remodelling that prevents vasodilation of the maternal spiral arteries does not alter the amount of blood and thus oxygen that reaches the placenta, but it increases the velocity of the blood flow from the maternal circulation (Burton *et al.*, 2009a). Furthermore, untransformed maternal spiral arteries retain their contractility resulting in intermittent blood flow (Scifres and Nelson, 2009). Increased velocity of the blood flow from the maternal circulation may cause damage to the villous structure and it could be responsible for the cystic lesions observed in IUGR placentae. Villous damage could extend to rupture of the chorionic villi, which would result in reduced supply of EVTs from CT columns (Burton *et al.*, 2009a). In addition, intermittent perfusion of the placenta is likely to cause oxidative stress and endoplasmic reticulum (ER) stress and thus activate the complement cascade resulting in tissue damage (Burton *et al.*, 2009a, Scifres and Nelson, 2009). This is supported by

evidence that there is inhibition of protein synthesis (Yung *et al.*, 2007) and increased activation of the complement cascade in IUGR placentae (Scifres and Nelson, 2009).

There is accumulating evidence that IUGR is associated with increased apoptosis in the ST. Histological examination of placental sections from IUGR pregnancies demonstrated that there is increased caspase activity in the IUGR placenta (Ishihara *et al.*, 2002, Smith *et al.*, 1997). Furthermore, the increased cell death in the IUGR villi is not balanced by changes in cell proliferation (Crocker *et al.*, 2004, Heazell *et al.*, 2008) suggesting that increased cell death could be partly responsible for the smaller size of the IUGR placenta. Ischaemia reperfusion injury caused as a result of insufficient spiral artery remodelling may contribute to the increased apoptosis in IUGR placental villi. *In vitro* experiments have demonstrated that IUGR CTs show increased susceptibility to apoptosis in response to hypoxia or hypoxia and reoxygenation (Crocker *et al.*, 2004). The increased apoptotic susceptibility of IUGR CTs appears to be mediated by increased p53 activity (Heazell *et al.*, 2008, Levy *et al.*, 2002) and increased expression of the pro-apoptotic molecule Mtd-1 (Soleymanlou *et al.*, 2007, Soleymanlou *et al.*, 2005). In addition to increased apoptosis, there is also increased fibrin deposition at tips of IUGR villi, which is also thought to be a result of hypoxia-reoxygenation injury particularly due to increased complement activation (Scifres and Nelson, 2009). Fibrin deposits co-localise with the C5b-9 membrane attack complex (MAC) and this co-localisation is increased in IUGR ST (Rampersad *et al.*, 2008). Furthermore, MAC binds to trophoblasts *in vitro* and modulates trophoblast apoptosis and differentiation.

The increased apoptosis and fibrin deposition in IUGR villi are likely to result in reduction in the functional mass of the ST and may induce changes in ST permeability ultimately resulting in inadequate transplacental exchange. Furthermore, villous damage

may affect the metabolic function of the placenta and could impair energy-dependent placental functions, such as active transport, conversion of glucose to lactate, regulation of amino acid availability and the production and secretion of placental hormones, such as hCG, hPL and PGH. Changes in placental endocrine function could in turn result in defective adaptation of the maternal metabolism to pregnancy causing further complications (Huppertz and Peeters, 2005). It is therefore obvious that IUGR is associated with gross placental abnormalities that compromise placental function and thus fetal growth. Better understanding of the mechanisms that alter maternal and trophoblast cell behaviour in IUGR can help to develop therapeutic strategies for this pregnancy complication.

1.2   Thyroid hormones

In humans, the thyroid hormones (THs) are synthesised in the thyroid gland and released into the circulation. THs have an impact on almost all tissues via their effects on the regulation of cell differentiation, growth and metabolism (Yen, 2001). The role of THs can vary in different tissues. THs affect bone growth and development by regulating the activity of osteoblast and osteoclast cells. In the heart, THs have effects on cardiac function and can regulate cardiac output. THs regulate lipolysis, lipogenesis and oxidative processes in the liver and they control the development and function of white and brown adipose tissue. Regulation of TH availability is essential for brain development both *in utero* and during the neonatal period. THs affect the differentiation of cells in the cerebral cortex, the visual and auditory cortex, the hippocampus and the cerebellum (Yen, 2001). TH availability is thus essential for the regulation of the differentiation and growth of many tissues.

1.2.1   Mechanisms controlling TH action

The cellular supply of THs is regulated by the circulating concentration of free THs, the expression of TH transporters at the plasma membrane of target cells, the activity of deiodinase enzymes and the expression of TH receptors (TRs) that mediate TH action at the target tissues.

1.2.1.1   Circulating concentrations of THs

The synthesis and secretion of THs is regulated by the hypothalamus-pituitary-thyroid axis (HPT axis) via a negative feedback system (**Figure 1-4**). Thyrotropin releasing hormone (TRH) is a tripeptide synthesised by the paraventricular nucleus of the

hypothalamus that binds to TRH receptors on thyrotrope cells in the pituitary and stimulates the secretion of thyroid stimulating hormone (TSH). TSH binding to TSH receptors that are expressed in thyroid follicular cells increases the expression of the Na^+/I^- symporter (NIS), of thyroglobulin (Tg) and of the enzyme thyroid peroxidase (TPO), that are all required for TH synthesis. NIS facilitates the transport of iodide into the thyroid, while TPO oxidises iodide in the thyroid in the presence of hydrogen peroxide, which is generated from oxygen in a reaction catalysed by NADPH oxidases. The oxidised form of iodide is incorporated in the tyrosine residues of Tg resulting in the production of monoiodinated (MIT) and diiodinated (DIT) tyrosine residues that are enzymatically coupled to form the THs thyroxine (T_4) and 3,3',5-tri-iodothyronine (T_3). The iodinated Tg is internalised from the apical surface of the thyroid follicular cell and is incorporated in lysosomes, where it undergoes proteolytic digestion causing the release of T_4, T_3, MIT and DIT into the cytoplasm of the cell. T_4 and T_3 are then released into the circulation from the basal surface of the follicular cell. The optimal levels of TH secretion are maintained since T_4 and T_3 regulate the secretion of TSH and TRH by a negative-feedback system. Most of the circulating TH is in the form of T_4 with total serum T_4 concentration being approximately 90 nM *versus* 2 nM of total serum T_3 (Yen, 2001). The majority of circulating THs are bound to proteins such as thyroxine binding globulin, albumin and transthyretin with the concentration of free circulating T_4 amounting to 0.03% of total serum T_4, whilst 0.3% of total serum T_3 is free. Only unbound THs can enter cells and generate a biological response (Yen, 2001).
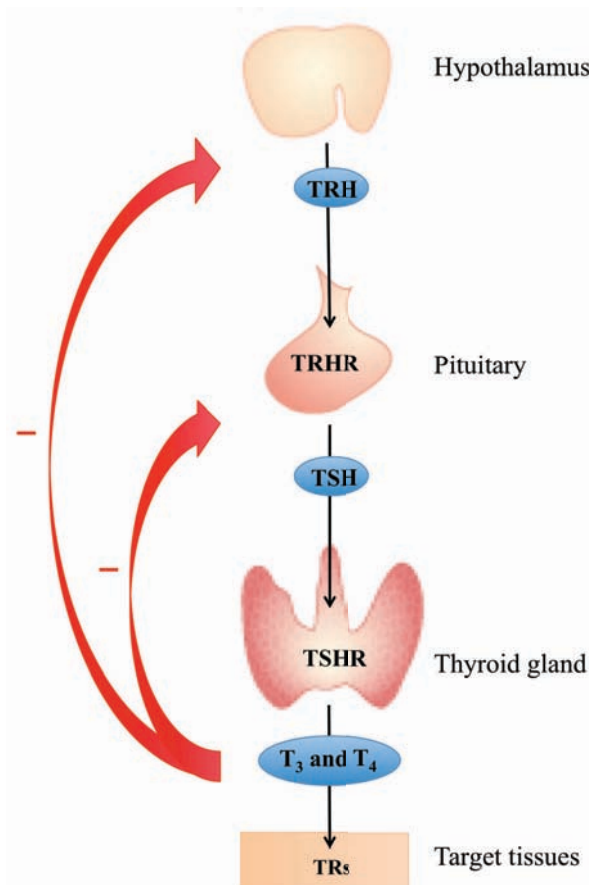
Figure 1-4: *The hypothalamus-pituitary-thyroid axis. The secretion of THs is regulated by a negative feedback system that involves the hypothalamus, pituitary and thyroid. The hypothalamus secretes thyrotropin releasing hormone (TRH) that binds to TRH receptors (TRHR) in the pituitary and promotes the secretion of thyroid stimulating hormone (TSH). TSH binds to TSH receptors (TSHR) in the thyroid gland and promotes the expression of the molecules necessary for the production of THs via iodination of tyrosine residues. THs (T_4 and T_3) are then released into the circulation. THs negatively regulate the secretion of TRH and TSH thus controlling TH production.*

1.2.1.2   TH transport

Although it was previously thought that due to their lipophilic nature THs entered the cells by passive diffusion, it is now known that transporter proteins are required to facilitate the passage of THs into and out of cells (Hennemann *et al.*, 2001). Several proteins that facilitate TH transport have been identified to date and they belong to

different families including the monocarboxylate transporter (MCT) family, L-Type Amino acid transporters (LATs), organic anion transporting polypeptides (OATPs) and the Na^+-taurocholate cotransporting polypeptide (NTCP). These TH transporters differ in their tissue distribution, substrate specificity and affinity for TH transport. The characteristics of the TH transporters identified to date are described in the following paragraphs and summarised in **Table 1**. As shown in **Table 1**, the reported Km values for the TH transporters indentified to date are in the nM or μM range. However, the circulating concentration of free T_4 and free T_3 is in the pM range. The high Km values of the TH transporters compared with the circulating concentration of free THs could indicate that *in vivo* TH transport cannot be saturated by circulating THs and that there is a linear relationship between the circulating concentration of TH and the amount of TH transported into the cells (Hennemann *et al.*, 2001). TH transport could be regulated by the number of TH transporters present at the cell membrane and by the circulating concentration of inhibitors for TH transport (Hennemann *et al.*, 2001). Furthermore, the reported Km values of the TH transporters for T_4 and T_3 were determined in experiments with *Xenopus* oocytes and therefore no intracellular THs were present initially. As a result, these are "zero trans entry" kinetics and they may not be representative of the *in vivo* condition. That TH uptake requires transporters is strongly suggested by the finding that it is energy-dependent both *in vitro* and *in vivo*, (Hennemann *et al.*, 2001) and, as discussed in section 1.4, patients affected by mutations of the TH transporter, MCT8 have altered circulating concentrations of THs (elevated serum T_3 and low serum T_4) (Dumitrescu *et al.*, 2004, Friesema *et al.*, 2004). In mice, MCT8 knock-down results in the same changes in the serum concentration of THs (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007). This evidence strongly indicates that MCT8 has a role in TH transport *in vivo*.

1.2.1.2.1 MCT8 and 10

The MCT family consists of 14 members, of which four (MCT1-4) have been characterised as monocarboxylate transporters. Two members of the MCT family, MCT8 and MCT10, that share 49% amino acid identity, have been identified as TH transporters (Friesema *et al.*, 2003, Friesema *et al.*, 2008). MCT8 and MCT10 have similar structures: they consist of 12 hydrophobic transmembrane domains (TMD) (Kim *et al.*, 2002, Lafreniere *et al.*, 1994) and have a leucine zipper-like motif located in a transmembrane region (Kim *et al.*, 2002, Lafreniere *et al.*, 1994) and a N-terminal sequence rich in proline, glutamic acid, serine and threonine repeats (PEST domain) (Lafreniere *et al.*, 1994, Ramadan *et al.*, 2006). The structure of MCT8 is illustrated in **Figure 1-5**. The predicted molecular mass of the human MCT8 and MCT10 proteins are 67 kDa (Lafreniere *et al.*, 1994) and 56 kDa (Kim *et al.*, 2001), respectively.
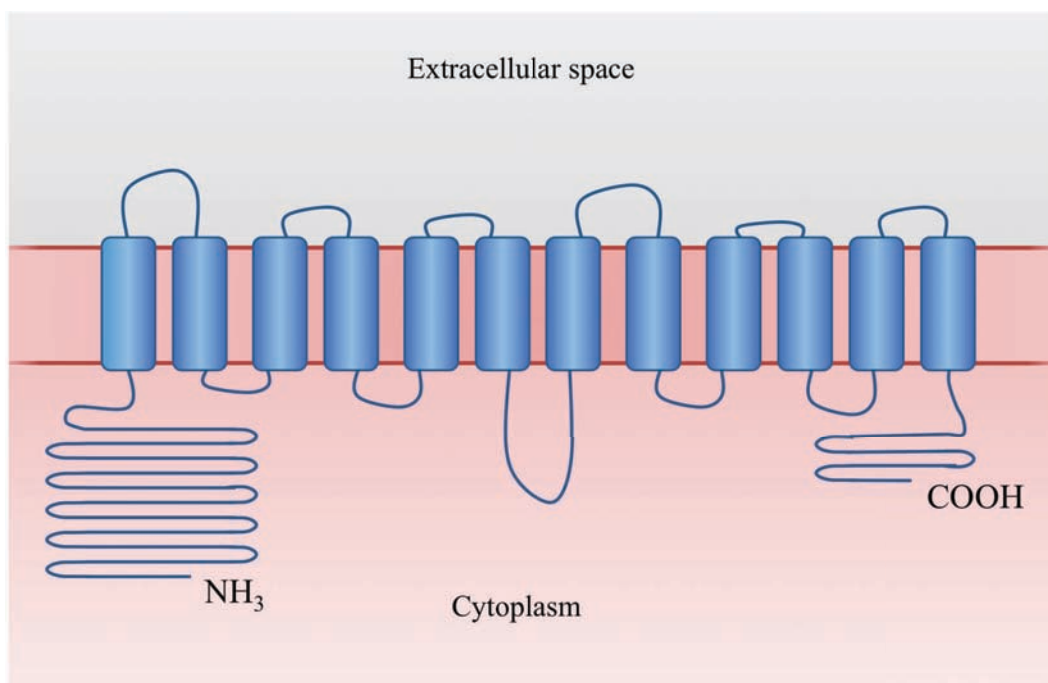
Figure 1-5: *Simplified diagram of the MCT8 protein structure. The MCT8 protein has 12 transmembrane domains shown as blue rods. Both the N terminus and the C terminus of the MCT8 protein are located intracellularly.*

MCT8 was first characterised by Lafreniere *et al.* while investigating genes associated with X chromosome inactivation (Lafreniere *et al.*, 1994). The authors identified a gene (official gene symbol: *SLC16A2*) that was only expressed from the active X chromosome and encoded for a protein that shared sequence homology and structural similarities with transporter proteins and they designated it XPCT for X-linked PEST containing transporter. It was subsequently demonstrated by Friesema *et al.* that MCT8 facilitates the transport of THs with a preference for T_3 over T_4 (Friesema *et al.*, 2003, Friesema *et al.*, 2006). Transfection of rat MCT8 in *Xenopus* oocytes resulted in a ten-fold increase in the uptake of T_3 (Km: 4.0 μM), T_4 (Km: 4.7 μM) and their metabolic derivatives rT_3 and T_2 (Km: 2.2 μM). Transport of T_4 was strongly inhibited by L-T_4, D-T_4, L-T_3, D-T_3, 3,3',5-triiodothyroacetic acid (Triac), *N*-bromoacetyl-T_3 (BrAcT_3) and by the organic anion bromosulphophthalein (BSP), whilst T_3 transport was less affected by these inhibitors. T_3 transport was Na^+-independent, whilst the transport of T_4 was slightly inhibited in the absence of Na^+. The possible transport of other compounds by MCT8 was investigated and it was found that MCT8, unlike monocarboxylate transporters, does not transport lactate. Furthermore, MCT8 does not facilitate the transport of leucine or of the aromatic amino acids phenylalanine, tyrosine or tryptophan (Friesema *et al.*, 2003). Transfection of human choriocarcinoma JEG-3 and simian kidney fibroblast-like COS-7 cells with human MCT8 also increased TH transport and stimulated intracellular TH metabolism by deiodinase enzymes (Friesema *et al.*, 2008, Friesema *et al.*, 2006). Furthermore, co-transfection of simian kidney trophoblast-like COS-1 cells with MCT8 and the cytosolic protein *μ*-crystallin (CRYM) that binds to intracellular THs, demonstrated that MCT8 facilitates both TH uptake and efflux (Friesema *et al.*, 2008). MCT8 mRNA is highly expressed in adult liver, kidney, heart, lung, and placenta and in adult and fetal brain (Chan *et al.*, 2006, Friesema *et al.*, 2003, Lafreniere *et al.*, 1994,

Roberts *et al.*, 2008). Furthermore, the presence of MCT8 protein has been demonstrated in the placenta and in adult and fetal brain (Chan *et al.*, 2006, Roberts *et al.*, 2008).

The human MCT10 (*SLC16A10*), also known as T-type amino acid transporter-1 (TAT1), was characterised in 2002 (Kim *et al.*, 2002). MCT10 was initially identified as a system T amino acid transporter that facilitated Na^+-independent transport of aromatic amino acids (Kim *et al.*, 2001, Kim *et al.*, 2002). It was later reported that MCT10 functions as a net efflux pathway for aromatic amino acids (Ramadan *et al.*, 2006). Although it was previously reported that MCT10 does not mediate the transport of THs (Kim *et al.*, 2001, Kim *et al.*, 2002), in a recent study, Friesema *et al.* demonstrated that transfection of COS-1 cells with MCT10 caused a marked stimulation of cellular T_3 and T_4 uptake and increased intracellular TH metabolism (Friesema *et al.*, 2008). Co-transfection with CRYM demonstrated that MCT10 also facilitates efflux of T_3 and T_4. T_3 uptake by MCT10-transfected COS-1 cells was inhibited in the presence of the aromatic amino acids phenylalanine, tyrosine and tryptophan. However, T_3 efflux was not affected by these inhibitors (Friesema *et al.*, 2008). MCT10 mRNA is highly expressed in adult human intestine, kidney, heart and placenta (Kim *et al.*, 2002).

1.2.1.2.2 LAT1 and 2

LAT1 and 2, also known as system L, are members of the heterodimeric amino acid transporter (HAT) family. They consist of the heavy chain 4F2hc (*SLC3A2*), also known as CD98, that can form disulphide bonds with the light chain LAT1 (*SLC7A5*) or LAT2 (*SLC7A8*) (Pineda *et al.*, 1999, Prasad *et al.*, 1999, Rossier *et al.*, 1999, Yanagida *et al.*, 2001). Human LAT1 and LAT2 share 50% amino acid identity and the predicted molecular mass of the LAT1 and LAT2 proteins are 55 kDa and 58 kDa, respectively.

Both proteins are predicted to have 12 TMDs and their N and C termini are likely to be located intracellularly (Pineda *et al.*, 1999, Prasad *et al.*, 1999, Rossier *et al.*, 1999). Heterodimers of LAT1/4F2hc and LAT2/4F2hc facilitate the Na^+-independent transport of neutral amino acids (Verrey, 2003). It has been shown that the system L can facilitate both amino acid uptake and efflux and thus provides a transport system for amino acid exchange (Pineda *et al.*, 1999, Rossier *et al.*, 1999, Yanagida *et al.*, 2001). LAT1 mRNA is expressed widely including human liver, kidney, heart, bone marrow, brain, testis, placenta and skeletal muscle (Prasad *et al.*, 1999, Yanagida *et al.*, 2001), whilst LAT2 is primarily expressed in the human kidney, intestine and placenta (Rossier *et al.*, 1999). Based on the localisation of LAT1 and LAT2 mRNA, it has been postulated that LAT1 is responsible for cellular exchange of amino acids, whilst LAT2 is involved in epithelial re-absorption of amino acids in the kidneys and intestine (Pineda *et al.*, 1999, Rossier *et al.*, 1999).

The involvement of the system L in TH transport was recognised following reports that amino acid transport by system L was inhibited by T_3 in a competitive manner (Taylor and Ritchie, 2007). The ability of the system L to facilitate transport of iodothyronines as well as amino acids was investigated using *Xenopus* oocytes, which have negligible expression of system L, and the choriocarcinoma cell line BeWo, which has high endogenous expression of system L, as models (Friesema *et al.*, 2001, Ritchie *et al.*, 1999, Ritchie *et al.*, 2003, Ritchie and Taylor, 2001). System L-mediated transport of THs was first demonstrated when Ritchie *et al.* co-injected *Xenopus* oocytes with 4F2hc and IU12, the *Xenopus* homologue of mammalian LAT1 (Ritchie *et al.*, 1999). The authors reported that 4F2hc/IU12 co-expression induced saturable, Na^+-independent transport of T_3 and T_4 that was inhibited by rT_3, and tryptophan, but not by Triac, a TH

analogue lacking the amino acid moiety. Furthermore, it was shown that T_3 inhibits tryptophan uptake in a competitive manner. Following injection of *Xenopus* oocytes with human LAT1 and 4F2hc, Friesema *et al.* confirmed the ability of human LAT1/4F2hc heterodimers to transport THs, with a preference for T_3 over T_4 and reported Km values of 0.8 μM and 7.9 μM, respectively (Friesema *et al.*, 2001). The authors demonstrated reciprocal inhibition of TH transport by the amino acids phenylalanine, tyrosine, leucine and tryptophan and they reported that in contrast to system L, the Na^+-dependent system y^+L does not facilitate TH transport. The reciprocal inhibition of TH transport by amino acids was further confirmed by uptake studies using the choriocarcinoma cell line, BeWo (Ritchie and Taylor, 2001). In a subsequent study, Ritchie *et al.* used *Xenopus* oocytes co-transfected with 4F2hc/IU12 and cDNA encoding for the TH receptor dimer TR/RXR under the control of a luciferase promoter, to demonstrate that system L-mediated TH transport is important for nuclear accumulation of T_3 and activation of TH receptors (Ritchie *et al.*, 2003).

1.2.1.2.3   OATPs

The OATP family (*SLCO*) includes eleven plasma membrane transporters in humans, all containing twelve TMDs. OATPs show wide tissue distribution and substrate specificity (Hagenbuch, 2007). They mediate Na^+-independent transport of a variety of amphipathic organic compounds, such as bile acids, steroid hormones and their conjugates, linear and cyclic peptides, prostaglandins, drugs and xenobiotics (Hagenbuch, 2007).

Of the eleven human OATPs, seven have been reported to facilitate the transport of THs. These transporters are OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP3A1,

OATP4A1 and OATP4C1 and they differ in their tissue distribution and substrate specificity (Hagenbuch, 2007). OATP1A2 is a 670 amino acids-long protein that exists in different glycosylation states. It has a wide tissue distribution including the blood-brain barrier, kidney, lung, placenta and testis (Fujiwara *et al.*, 2001, Kullak-Ublick *et al.*, 2001, Patel *et al.*, 2003). In studies using *Xenopus* oocytes injected with OATP1A2, it was demonstrated that OATP1A2 mediates the transport of T_4 (Km: 8.0 μM), T_3 (Km: 6.5 μM) and rT_3 (Hagenbuch, 2007). OATP1B1 and 1B3 are liver-specific transporters with 80% amino acid identity and can both be glycosylated (Abe *et al.*, 1999, Kullak-Ublick *et al.*, 2001). Using *Xenopus* oocytes, it was shown that both OATP1B1 and 1B3 also transport the THs, T_4 and T_3 (Kullak-Ublick *et al.*, 2001). OATP1C1 is exclusively expressed in the blood brain barrier and the choroid plexus (Pizzagalli *et al.*, 2002). It facilitates the transport of BSP, estrone-3-sulphate and estradiol-17β-glucuronide. Uptake studies using Chinese hamster ovary cells (CHO) overexpressing OATP1C1, demonstrated that OATP1C1 is the OATP transporter with the highest affinity for THs, since the estimated Km values for T_4 and T_3 transport were 90.4 nM and 127.7 nM, respectively (Pizzagalli *et al.*, 2002). OATP1C1 transports rT_3 but not T_2. The gene *OATP3A1* encodes for two splice variants with different tissue distributions: OATP3A1v1 that shows ubiquitous expression and OATP3A1v2 that is predominantly expressed in the brain and testis. Both proteins facilitate the transport of T_4 as shown in CHO cells (Huber *et al.*, 2007). OATP4A1 is a transporter with wide tissue distribution and narrow substrate specificity. OATP4A1 expression is higher in the heart, placenta, lung, liver, skeletal muscle, kidney and pancreas. OATP4A1 transports taurocholate, benzylpenicillin, steroid conjugates and the THs, T_4, T_3 (Km: 0.9 μM) and rT_3 (Fujiwara *et al.*, 2001). OATP4C1 is expressed predominantly in the kidney and it transports the cardiac glycosides digoxin and ouabain, methotrexate and cAMP. OATP4C1 facilitated cellular uptake of T_4 and T_3

(Km: 5.9 μM) (Mikkaichi *et al.*, 2004). These molecules are likely to play a tissue-specific role in the transport of THs and other substrates. Their relative contribution for TH transport is likely to depend on the circulating concentration of THs and other substrates that could inhibit TH transport and their co-localisation with other transporters with different affinities for THs and other substrates.

1.2.1.2.4   NTCP

NTCP is facilitating Na^+-dependent transport of bile acids in the liver. NTCP, which has seven TMDs, is exclusively expressed in hepatocytes and transports all physiological bile acids including taurocholate, glycocholate, taurochenodeoxycholate and tauroursodeoxycholate (Trauner and Boyer, 2003). In addition to bile acids, NTCP facilitates the transport of other compounds such as oestrogen conjugates, BSP, dehydroepiandrosterone sulphate and THs.

Table 1-1: *Characteristics of TH transporters*

Transporter	Tissue expression (human)	K_m		Other substrates
		T_4	T_3	
MCT8 (SLC16A2)	Liver, kidney, brain, blood-brain barrier, heart, lung, placenta (Chan *et al.*, 2006, Lafreniere *et al.*, 1994, Roberts *et al.*, 2008)	Rat MCT8: 4.7 μM (Friesema *et al.*, 2003)	Human MCT8: 0.86 μM (Kinne *et al.*, 2009) Rat MCT8: 4.0 μM (Friesema *et al.*, 2003)	Not reported
MCT10 (SLC16A19)	Intestine, kidney, heart, placenta (Kim *et al.*, 2002)	Not reported	Not reported	Aromatic amino acids (Kim *et al.*, 2001, Kim *et al.*, 2002, Ramadan *et al.*, 2006)
LAT1 (SLC7A5)	Liver, kidney, heart, bone marrow, brain, skeletal, muscle, testis, placenta (Prasad *et al.*, 1999, Yanagida *et al.*, 2001)	LAT1/4F2hc: 7.9 μM (Friesema *et al.*, 2001)	LAT1/4F2hc: 0.8 μM (Friesema *et al.*, 2001)	Neutral amino acids (Verrey, 2003, Yanagida *et al.*, 2001)
LAT2 (SLC7A8)	Kidney, intestine, placenta (Rossier *et al.*, 1999)	Not reported	Not reported	Neutral amino acids (Pineda *et al.*, 1999, Rossier *et al.*, 1999, Verrey, 2003)
OATP1A2 (SLCO1A2)	Blood-brain barrier, kidney, lung, testis, placenta (Kullak-Ublick *et al.*, 2001, Patel *et al.*, 2003)	8.0 μM (Hagenbuch, 2007)	6.5 μM (Hagenbuch, 2007)	Amphipathic organic compounds (Hagenbuch, 2007)
OATP1B1 (SLCO1B1)	Liver (Abe *et al.*, 1999, Kullak-Ublick *et al.*, 2001)	Not reported	Not reported	Amphipathic organic compounds (Hagenbuch, 2007)
OATP1B3 (SLCO1B3)	Liver (Abe *et al.*, 1999, Kullak-Ublick *et al.*, 2001)	Not reported	Not reported	Amphipathic organic compounds (Hagenbuch, 2007)
OATP1C1 (SLCO1C1)	Blood-brain barrier, choroid plexus and testis (Hagenbuch, 2007, Pizzagalli *et al.*, 2002)	90.4 nM (Pizzagalli *et al.*, 2002)	127.7 nM (Pizzagalli *et al.*, 2002)	Amphipathic organic compounds (Hagenbuch, 2007)
OATP3A1 (SLCO3A1)	Variant 1: ubiquitous expression. Variant 2: Brain and testis (Huber *et al.*, 2007)	Not reported	Not reported	Amphipathic organic compounds (Hagenbuch, 2007)
OATP4A1 (SLCO4A1)	Heart, placenta, lung, liver, skeletal muscle, kidney, pancreas (Fujiwara *et al.*, 2001)	Not reported	0.9 μM (Fujiwara *et al.*, 2001)	Taurocholate, benzylpenicillin, steroid conjugates (Fujiwara *et al.*, 2001)
OATP4C1 (SLCO4C1)	Kidney (Mikkaichi *et al.*, 2004)	Not reported	5.9 μM (Mikkaichi *et al.*, 2004)	Digoxin, ouabain, methotrexate and cAMP (Mikkaichi *et al.*, 2004)
NTCP (SLC10A1)	Liver (Trauner and Boyer, 2003)	Not reported	Not reported	Bile acids, oestrogen conjugates (Trauner and Boyer, 2003)

1.2.1.3   TH metabolism

1.2.1.3.1   Deiodination

The major pathway that controls TH metabolism is deiodination by iodothyronine deiodinases (**Figure 1-6**). The deiodinases are selenocysteine-containing enzymes that remove iodine moieties from the outer (phenolic) or the inner (tyrosine) ring on THs. T_3 is produced locally by the outer-ring deiodination (ORD) of T_4, whilst inner-ring deiodination (IRD) of T_4 and T_3 convert them to their inactive metabolites rT_3 and T_2 (figure). It is estimated that peripheral T_3 production by T_4 deiodination accounts for approximately 80% of the T_3 produced daily (Bianco and Kim, 2006). Therefore, deiodinases play a major role in determining the availability of the active TH ligand, T_3, within tissues. There are three deiodinase subtypes, type I (D1), type II (D2) and type III (D3) (Bianco and Kim, 2006). All types of deiodinases form homodimers and are integral membrane proteins (Gereben *et al.*, 2008). The expression of the deiodinase genes *Dio1*, *Dio2* and *Dio3*, encoding for D1, D2 and D3 proteins respectively, is regulated by different factors including cAMP, glucocorticoids and cytokines and by THs themselves. T_3 upregulates the expression of D1 and D3, whilst it downregulates D2 expression. Furthermore, T_4 induces the ubiquitination of D2 and thus downregulates its activity (Gereben *et al.*, 2008).

D1 is expressed in many tissues including the human liver, kidney, thyroid gland, pituitary and intestine. It is localised in the plasma membrane with its active site in the cytoplasm. D1 has both ORD and IRD activity and can thus catalyse the conversion of T_4 to either T_3 or reverse T_3 (rT_3) and the deiodination of rT_3 to diiodothyronine (T_2). However, D1 is not as efficient as D2 and D3 in the deiodination of T_4, since its Km for

T_4 is in the µM range compared with D2 and D3 that have a Km in the nM range for T_4. It is thought that D1 plays a balancing role in maintaining optimal T_3 levels in cases of altered thyroid function (Bianco and Kim, 2006, Gereben *et al.*, 2008).

D2 has ORD but not IRD activity and can thus catalyse the monodeiodination of T_4 to T_3 and the conversion of rT_3 to T_2. D2 is the primary activating enzyme in tissues and is thus the major source of intracellular and plasma T_3 (Salvatore *et al.*, 1996). D2 is expressed widely including in the human brain, anterior pituitary, thyroid, brown fat, skeletal muscle, heart and is present at low levels in the placenta (Croteau *et al.*, 1996, Kuiper *et al.*, 2005). D2 is an ER-resident protein with its N-terminus in the ER lumen and its catalytic domain in the cytoplasm (Gereben *et al.*, 2008).

D3 is the deactivating enzyme that catalyses the monodeiodination of T_4 to rT_3 and of T_3 to T_2 by IRD (Gereben *et al.*, 2008). It is present in the placenta, pregnant uterus, fetal tissues and in the brain (Chan *et al.*, 2003, Chan *et al.*, 2002, Gereben *et al.*, 2008, Koopdonk-Kool *et al.*, 1996, Kuiper *et al.*, 2005). D3 has been localised in the plasma membrane and it has been shown that it is internalised into clathrin-coated endosomes and is recycled between the plasma membrane and early endosomes (Baqui *et al.*, 2003). There is evidence that the catalytic subunit of D3, when membrane-bound, is located extracellularly thus allowing the rapid inactivation of circulating T_4 and T_3 (Baqui *et al.*, 2003, Gereben *et al.*, 2008), however this view has been challenged by recent findings that MCT8 increases D3-mediated deiodination (Gereben *et al.*, 2008). D3 is thought to be important in limiting the exposure of developing fetal tissues to T_3 (Gereben *et al.*, 2008). The physiological roles of rT_3 and T_2 have not been elucidated. Since they cannot bind to TRs it was thought that they are inactive metabolites, however recent evidence

suggests that rT_3 plays a role in actin polymerisation and T_2 is involved in mitochondrial

respiration (Davis *et al.*, 2008, Wrutniak-Cabello *et al.*, 2001).
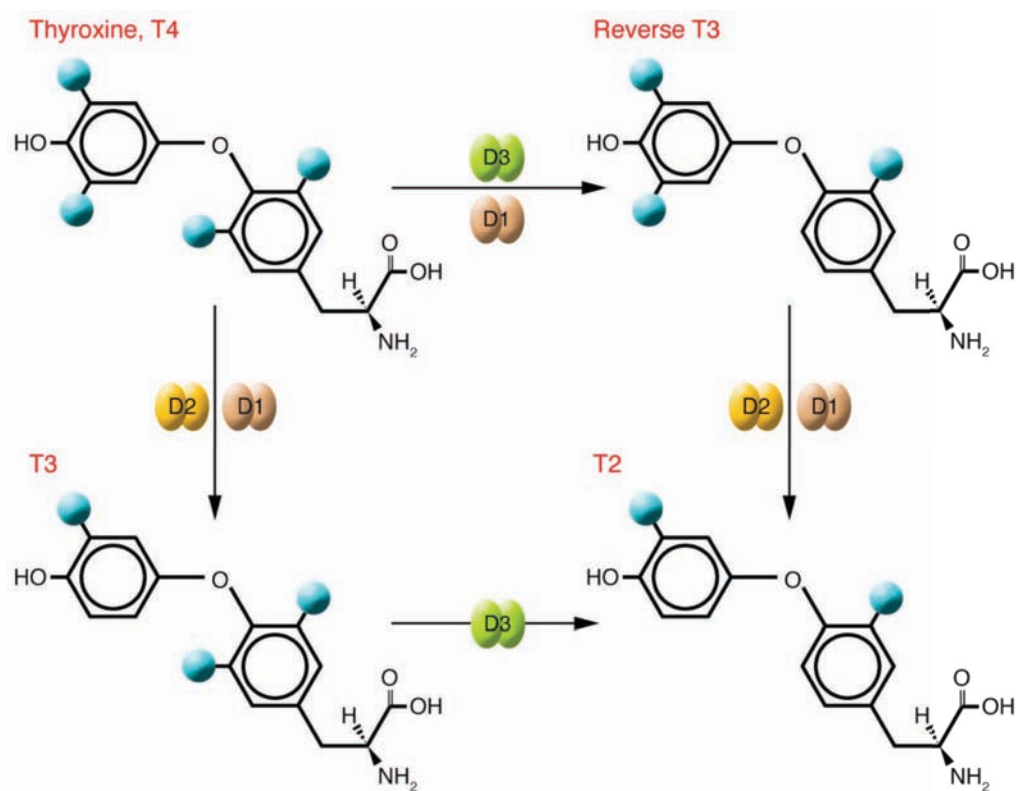
Figure 1-6: *Deiodination of THs. Intracellular availability of the different TH forms is regulated via the activity of deiodinase enzymes. Deiodinase 1 (D1) has both inner and outer ring deiodination activity and can thus convert T_4 to either T_3 or rT_3 and it can facilitate the conversion of rT_3 to T_2. Deiodinase 2 (D2) facilitates the conversion of T_4 to T_3 and rT_3 to T_2 via outer ring deiodination. Deiodinase 3 (D3) has inner ring deiodination activity and converts T_4 to rT_3 and T_3 to T_2. Iodine moieties are represented by blue spheres. Figure from (Bianco and Kim, 2006).*

1.2.1.3.2 Alternate pathways

Although deiodination is the major metabolic pathway that controls TH

metabolism, THs are also metabolised by sulphation, glucuronidation, deamination,

decarboxylation and ether-link cleavage (**Figure 1-7**).

TH sulphation is the transfer of a sulphonate group from the sulphonate donor, 3'-phosphoadenosine 5-phosphosulphate to the phenolic hydroxyl group of THs and is catalysed by sulphotransferase enzymes (Wu *et al.*, 2005). Sulphotransferases (SULTs) are cytosolic enzymes composed of two subunits and they belong to three superfamilies: SULT1, SULT2 and SULT3. Sulphotransferases have overlapping substrate specificity and they are not specific for THs. The human sulphotransferases that are active towards THs are SULT1A1, SULT1A3, SULT1A5, SULT1B1, SULT1B2, SULT1C1, SULT1E1 and SULT2A1. They are expressed in the human liver, thyroid gland and uterus. T_3 and rT_3 are converted to T_3S and T_2 to T_2S and their sulphation accelerates their deiodination. On the other hand, T_4 is a poor substrate for sulphotransferases and following its sulphation to T_4S, it cannot be deiodinated to T_3S. Sulphated THs can be hydrolysed to their precursors in liver, brain and other tissues. It is therefore postulated that T_3S functions as a reservoir for T_3 (Wu *et al.*, 2005).

Glucuronidation is the phenolic conjugation of THs with glucuronic acid that is catalysed by uridine diphosphate-glucuronosyltransferase (UGT), thus converting T_4 to T_4G. The glucuronidation of T_3 is minimal in humans (Wu *et al.*, 2005). Glucuronidation occurs primarily in the human liver and kidney by UGT1A1 and UGT1A9, respectively and precedes the biliary or fecal excretion of THs. In the intestinal lumen, T_4G is converted to T_4 via a reaction catalysed by β-glucuronidase. Therefore, it is thought that T_4G functions as a reservoir for T_4 (Wu *et al.*, 2005).

Other TH metabolites arise from the deamination or decarboxylation of the alanine side chain of THs (Wu *et al.*, 2005). Deamination is catalysed by L-amino acid oxidase (expressed in the kidney) and TH aminotransferase (expressed in the liver and kidney) and leads to the formation of the acetic analogues, Triac and Tetrac. Triac and Tetrac have

thyromimetic activity and can regulate the secretion of TSH. Furthermore, Triac can bind

to TRs with a higher affinity than T_3, with a preference for TRβ1. Tetrac can be converted

to Triac by D2. Decarboxylation of the alanine side chain of THs leads to the formation of

Tetram, Triam, T_1am or T_0am. Although the *in vivo* occurrence of Tetram and Triam has

not been confirmed, T_1am and T_0am were found in the rodent brain indicating that they

may have a biological role (Wu *et al.*, 2005).

Cleavage of the ether-link of THs by peroxidases is thought to be a minor pathway

in TH metabolism (Wu *et al.*, 2005). Ether-link cleavage leads to the production of DIT

and iodine. The circulating concentration of DIT is between 0.02 and 0.55 nM and it is

thought that DIT is secreted by the thyroid rather than produced by cleavage of THs.

However, it has been shown that during infection the circulating concentration of DIT

increases dramatically to > 5 nM and it has been postulated that ether-link cleavage of

THs may play a role in bacterial clearing by leukocytes (Wu *et al.*, 2005).
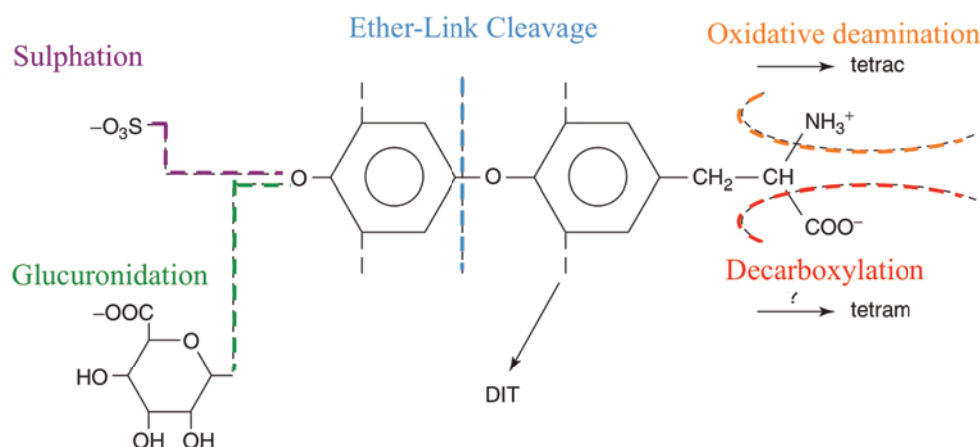
Figure 1-7: *Alternate pathways of TH metabolism. THs can be converted to different metabolites by sulphation, glucuronidation, ether-link cleavage, oxidative deamination and decarboxylation. The sulphation of THs is catalysed by sulphotransferase enzymes that transfer a sulphonate group to the phenolic hydroxyl group of THs. Glucuronidation involves the phenolic conjugation of THs with glucuronic acid and is catalysed by uridine diphosphate glucuronosyltransferase. Ether-link cleavage of THs is catalysed by peroxidase enzymes and results in the production of diiodotyrosine (DIT). Oxidative deamination of T_4 results in the production of Tetrac, whilst decarboxylation generates Tetram. Figure adapted from (Wu et al., 2005).*

1.2.1.4   TH receptors

T$_3$, the active TH ligand, exerts its biological function by binding to TH receptors (TRs), ligand-regulated transcription factors that belong to the nuclear hormone receptor superfamily. There are two major TR isoforms (TRα and TRβ) and they are encoded by two separate genes, the *c-erbAα* gene and the *c-erbAβ* gene respectively, which are cellular homologs of the viral oncogene protein V-*erbA* (Yen, 2001). Alternative splicing of TRα generates two different protein species, TRα1 and TRα2, whilst alternative splicing of the TRβ gene generates TRβ1 and TRβ2. TRα1, TRβ1 and TRβ2 are known to bind T$_3$. In contrast, TRα2 does not bind T$_3$ and may therefore inhibit the function of the other TRs by competitive binding to the TRE site (Yen, 2001). TRα1 and TRβ1 are ubiquitously expressed. In contrast, TRβ2 is only found in specific tissues, namely in the anterior pituitary gland, in the hypothalamus and in the developing brain. TR protein structure consists of an amino-terminal region, a central DNA-binding domain that contains two zinc fingers, a lysine-rich hinge region that contains a nuclear localisation signal and is implicated in TR binding with cofactors and a carboxy-terminal ligand-binding domain that is essential for TH binding and for TR dimerisation (Yen, 2001).

TRs exert their function via binding to TH response elements (TREs) upstream of TH-regulated genes. TREs are composed of hexamer half-sites with a consensus sequence of (G/A)GGT(C/G)A that may exist as direct repeats, palindromes or inverted palindromes. TRs bind to TREs as monomers, homodimers or heterodimers with other auxiliary protein and initiate or repress gene expression. The major TR auxiliary proteins are retinoic acid X receptors (RXRs). TRs also form heterodimers with other auxiliary proteins including RAR, PRAP and VDR. TR/RXR heterodimers show enhanced binding to TREs compared with TR monomers or homodimers. Furthermore, TRs can be

phosphorylated thus increasing DNA-binding (Yen, 2001). TRs can modulate the expression of TH-regulated genes depending on the presence of their ligand, T_3. Unliganded TRs form complexes with corepressor molecules, such as NCoR, SMRT and SUN-CoR and they interact with the transcription machinery to repress the transcription of positively regulated genes. In contrast, the expression of some negatively regulated genes is upregulated by unliganded TR-corepressor complexes. In the presence of T_3, TRs complex with coactivator molecules such as SRC-1 and with transcription factors to activate the expression of positively regulated genes or inhibit the expression of negatively regulated genes (Yen, 2001).

Whilst T_3-binding to nuclear TRs is the classical pathway of TH action, it is now known that not all the effects of THs are mediated via intranuclear complexing of TRs and T_3 (Davis *et al.*, 2008, Furuya *et al.*, 2009). Non-genomic effects of THs are mediated via binding to extranuclear TRs. TRβ1 and truncated forms of TRα1 are found in the cytoplasm where they bind to T_3 and initiate protein kinase pathways (Davis *et al.*, 2008, Furuya *et al.*, 2009). Furthermore, a novel cell surface TR domain has been identified on integrin αVβ 3. T_4, and to a lesser extent T_3, binds to this receptor and causes the activation of protein kinases by phosporylation (Davis *et al.*, 2008). Since this receptor is located extracellularly, cellular entry of THs is not necessary for its activation. TH binding to extranuclear TRs enhances cell proliferation, motility and angiogenesis of tumour cells (Davis *et al.*, 2008). In addition, truncated forms of TRα1 are found in the mitochondria where they are thought to initiate T_3-dependent transcription of mitochondrial genes and partly mediate the influence of THs on mitochondrial oxygen consumption (Wrutniak-Cabello *et al.*, 2001). The genomic and non-genomic pathways of TH action are summarised in **Figure 1-8**.
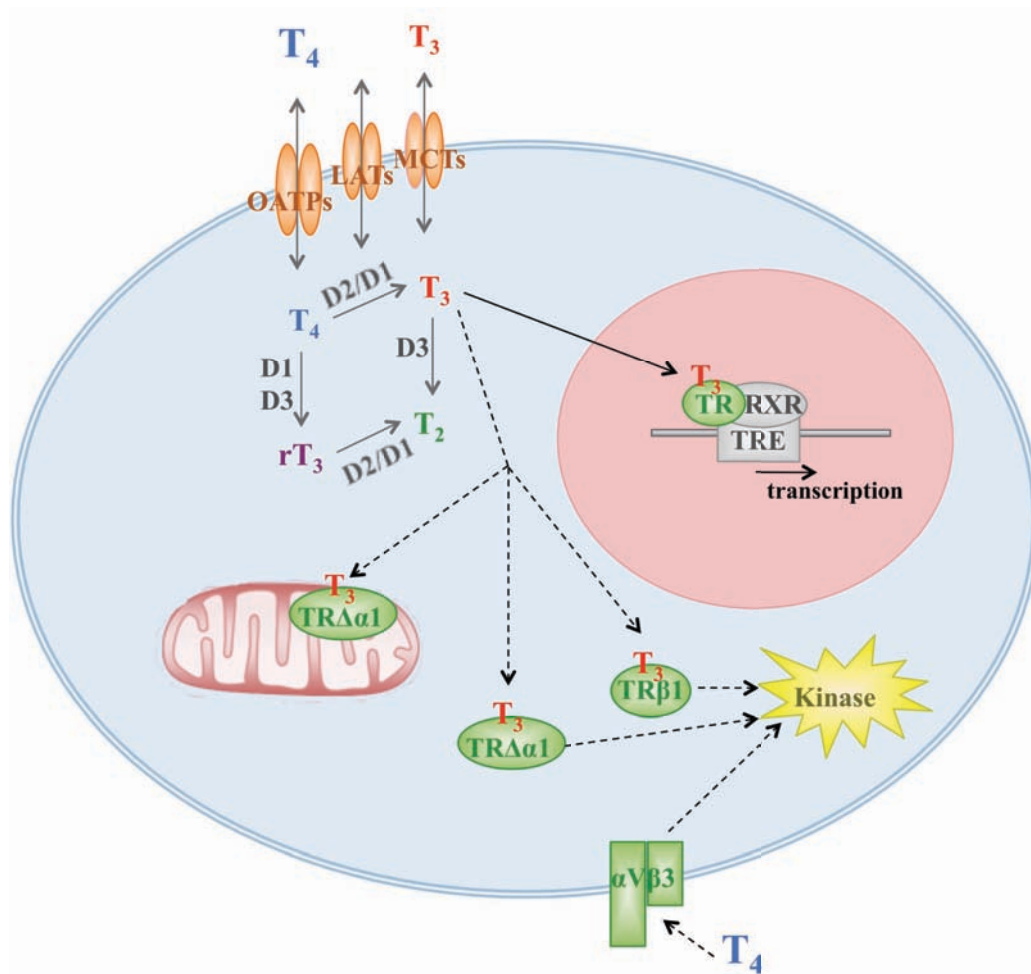
Figure 1-8: *Genomic and non-genomic pathways of TH action. THs enter the cells via TH transporters (MCTs, LATs or OATPs) and are metabolised via the deiodinase enzymes (D1, D2 and D3). Genomic TH action (solid black arrow) involves binding of the active ligand, T_3, to TH receptors (TRs) in the cell nucleus. TRs are nuclear transcription factors that heterodimerise with other nuclear receptors such as retinoic acid X receptor (RXR) and regulate gene transcrption. Non genomic pathways of TH action (dashed arrows) involve binding of T_3 to TRs outside the nucleus. A truncated form of TRα1 (TRΔα1) is located in the mitochondria. Binding of T_3 to this receptor regulates mitochondrial gene expression. TRβ1 and TRΔα1 are also found in the cytoplasm. T_3 binding to these receptors leads to the activation of kinase pathways. Furthermore, a new TR has been identified on the integrin αVβ3. T_4 binding to this receptor that is located extracellularly and does not require T_4 entry into the cell leads to the activation of kinase pathways.*

1.3 THs in pregnancy

Optimal levels of maternal THs are necessary for normal fetal development, particularly for the development of the central nervous system (CNS) (Haddow *et al.*, 1999, Morreale de Escobar *et al.*, 2004, Pop *et al.*, 2003). The transfer of maternal THs to the fetal circulation is regulated by the placenta thus preventing toxic concentrations of THs from reaching the developing fetus. As well as facilitating the transport of maternal TH to the fetal circulation, the human placenta is thought to be sensitive to the action of TH for the regulation of its metabolism, differentiation and development (Kilby *et al.*, 2005). The importance of TH availability on fetoplacental development is highlighted by the association of untreated maternal hyperthyroidism and subclinical hypothyroidism with pregnancy complications including miscarriages, pre-eclampsia, IUGR and stillbirths (Abalovich *et al.*, 2002, Casey *et al.*, 2005, LaFranchi *et al.*, 2005, Mestman, 2004).

1.3.1 Transplacental transport of THs

Previously the placenta was considered impermeable to maternal THs, partly because it expresses D3 that metabolises T_4 and T_3 to their inactive metabolites. However, there is now evidence that transfer of T_3 and T_4 occurs from the mother to the fetus since the first trimester (Calvo *et al.*, 2002, Calvo *et al.*, 1990, Contempre *et al.*, 1993, Vulsma *et al.*, 1989). After the onset of fetal TH production in the mid second trimester, THs in the fetal circulation are of both maternal and fetal origin (Chan *et al.*, 2009). Even though fetal TH production does not begin until the second trimester of pregnancy, THs are present in human embryonic cavities since the first trimester, indicating that maternal THs reach the developing fetus (Calvo *et al.*, 2002, Contempre *et al.*, 1993). Furthermore, at term T_4 was detected in the umbilical cord blood of infants with severe congenital

hypothyroidism that cannot produce their own THs, thus providing further proof for the transfer of maternal THs to the fetus during gestation (Vulsma *et al.*, 1989). When a rat model of congenital hypothyroidism was studied, it was demonstrated that infusion of T_4 into the mothers caused an increase in the concentration of T_4 and particularly T_3 in the fetal brain (Calvo *et al.*, 1990).

A number of factors may regulate the amount and the different forms of THs that are transported from the maternal to the fetal circulation. The human placenta expresses a range of transporters that can facilitate TH transport. The TH transporters that have been identified in the human placenta to date are MCT8 (Chan *et al.*, 2006), MCT10 (Park *et al.*, 2005), LAT1 (Okamoto *et al.*, 2002), LAT2 (Kudo and Boyd, 2001), OATP1A2 (Patel *et al.*, 2003) and OATP4A1 (Briz *et al.*, 2003, Patel *et al.*, 2003, Sato *et al.*, 2003). With the exception of MCT8, all TH transporters expressed in the placenta are involved in the transport of substrates other than THs. The relative contribution of each TH transporter to transplacental TH transport depends on their affinity for THs, competition by other substrates and their localisation within the placenta. The data available to date indicate that MCT8, LAT1 and OATP4A1 are localised in the ST, consistent with a role of these transporters for transplacental TH transport (Chan *et al.*, 2006, Okamoto *et al.*, 2002, Ritchie and Taylor, 2001, Sato *et al.*, 2003). The localisation of MCT10, LAT2 and OATP1A2 within the placenta has not been elucidated yet.

TH metabolism by deiodinase enzymes and to a lesser extent by sulphatase enzymes within the placenta further modulates TH availability. There is low sulphatase activity in the human placenta, suggesting that these enzymes do not play a major role in the metabolism of THs within the placenta (Chan *et al.*, 2009). In contrast, substantial D3 and to a lesser extent D2 activity is present in the human placenta from week 6 of

gestation and both mRNA expression and protein activity of D2 and D3 in the human placenta decline significantly with advancing gestation (Chan *et al.*, 2003, Koopdonk-Kool *et al.*, 1996). Therefore, maternal T_4 and T_3 are probably metabolised within the placenta, thus regulating the TH forms available for efflux to the fetal capillaries throughout gestation. Recently, it was demonstrated that the placenta synthesises and secretes the TH-binding protein transthyretin. Furthermore, it was shown that transthyretin binds to T_4 extracellularly and the complex is then internalised by placental explants (Landers *et al.*, 2009). T_4 binding to transthyretin protects it from inactivation by D3 and may thus play an important role in the supply of maternal T_4 to the fetus. In addition to expressing transporter proteins and deiodinase enzymes that facilitate TH transport and metabolism, human placental tissue has a high nuclear binding capacity for T_3 (Ka = 7.0×10^9 M^{-1}; capacity = 62.7 fmol T_3/mg DNA) (Ashitaka *et al.*, 1988, Banovac *et al.*, 1986). Furthermore, mRNA and protein of the T_3-binding TR isoforms, TRα1 and TRβ1, are expressed in the human placenta (Ashitaka *et al.*, 1988, Banovac *et al.*, 1986, Kilby *et al.*, 1998). Therefore, binding of T_3 to TRs within the placenta may further restrict the amount of T_3 available for transport to the fetal circulation.

It is thought that T_4 is the major TH crossing the placenta, which is supported by the correlation of the concentration of maternal and fetal serum total T_4 (Chan *et al.*, 2009, Morreale de Escobar *et al.*, 2004). However, there is evidence that CTs can also transport T_3 (Mitchell *et al.*, 1999, Ritchie and Taylor, 2001). The level of free T_4 in the maternal circulation increases moderately in the first trimester possibly as a mechanism of ensuring adequate supply of maternal THs to the fetoplacental unit. It is thought that this increase is triggered by hCG that is secreted by the placenta and has weak TSH-like activity (Chan *et al.*, 2009). The concentration of free T_4 in the fetal circulation increases throughout

gestation and in the third trimester it exceeds the concentration of maternal free T_4. In contrast, the fetal concentration of free T_3 remains lower than the maternal free T_3 throughout gestation (Chan *et al.*, 2009) (**Figure 1-9**). The TH metabolites, rT_3, T_4S and T_3S are also present in the fetal circulation.
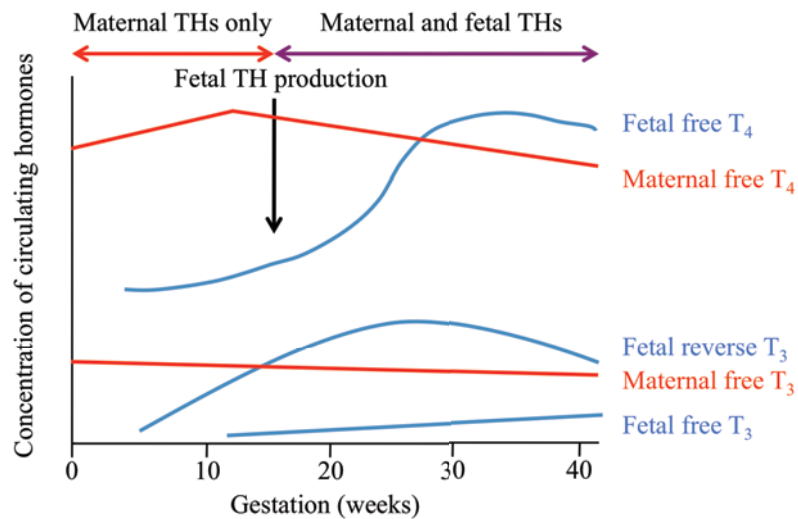
Figure 1-9: *Circulating maternal and fetal THs during human pregnancy. Before the onset of fetal TH production, around week 14 of human pregnancy, THs in the fetal circulation are exclusively of maternal origin. Following fetal TH production, there is a mixture of maternal and fetal THs in the fetal circulation. The concentration of free T_4 in the maternal circulation shows a moderate increase during the first trimester, whilst maternal free T_3 levels are relatively constant throughout pregnancy. Fetal free T_4 and reverse T_3 increase following the onset of fetal TH production and towards term free T_4 in the fetal circulation exceeds the concentration of free T_4 in the maternal circulation. The concentration of free T_3 in the fetal circulation remains lower than the circulation of free T_3 in the maternal circulation throughout pregnancy. Figure adapted from (Chan et al., 2009).*

1.3.2   Importance of THs for fetal development

The critical role of THs in growth and differentiation of many organs and thus overall development in human is well recognised (Chan and Kilby, 2000, Schwartz *et al.*, 1983). This is of particular significance for the development of the CNS since TH

deficiency has been associated with neurodevelopment morbidity. In many species, THs synchronise neuronal differentiation, polarity, synaptogenesis, cytoplasmic outgrowths and myelin formation via modulating the expression of TH-responsive genes (Bernal, 2005, Bernal and Nunez, 1995, Guadano-Ferraz *et al.*, 1999). In humans, the thyroid status of neonates and children has a significant long-term impact on their behaviour, locomotor ability, speech, hearing and cognition (Legrand, 1986). Development of different areas of the CNS has been associated with the timing and duration of TH deficiency, suggesting that there are critical periods during which various parts of the brain are sensitive to TH supply (Rovet *et al.*, 1992).

The association of maternal hypothyroidism with poorer neuropsychological outcome in the offspring demonstrates the critical role of TH in fetal brain development during pregnancy (Haddow *et al.*, 1999, Pop *et al.*, 2003, Pop *et al.*, 1999). Children of women with free T_4 levels below the fifth and tenth percentile at 12 weeks of gestation had significantly lower scores on the Bayley Psychomotor Developmental Index (PDI) scale at ten months of age, compared with children of mothers with higher free T_4 levels (Pop *et al.*, 1999). Furthermore, there was a positive correlation between maternal free T_4 levels and PDI scores. In a three-year follow-up study, children of women with hypothyroxinaemia at 12 weeks of gestation had delayed mental and motor functions compared with matched controls (Pop *et al.*, 2003). These data suggest that low maternal plasma free T_4 concentration during early pregnancy is an important risk factor for impaired development. Further evidence for the importance of THs on fetal brain development was provided by a study using rats where maternal hypothyroxinaemia during early pregnancy resulted in altered fetal brain histogenesis and cytoarchitecture (Lavado-Autric *et al.*, 2003).

The sensitivity of the developing brain to TH is indicated by the presence of molecules involved in TH entry, metabolism and action in the fetal brain. The fetal brain has the potential for TH uptake as demonstrated by the expression of the TH transporters LAT1, OATP1C1 and MCT8 (Friesema *et al.*, 2003, Hagenbuch, 2007, Heuer, 2007, Lafreniere *et al.*, 1994, Taylor and Ritchie, 2007, Yanagida *et al.*, 2001). OATP1C1 is expressed in the blood-brain barrier, the route by which THs preferentially enter the brain, and is thus thought to play an important role in the delivery of THs, mainly T_4, to the brain (Bernal, 2005, Hagenbuch, 2007, Pizzagalli *et al.*, 2002). MCT8 expression was studied in the murine CNS (Heuer *et al.*, 2005). High levels of MCT8 mRNA were detected in the choroid plexus and in other brain regions including the olfactory bulb, cerebral cortex, hippocampus and amygdala. Moderate levels of MCT8 mRNA were found in the striatum and cerebellum and low levels in a few neuroendocrine nuclei. It was therefore postulated that MCT8 playa a critical role in facilitating T_3 uptake by neurons, where T_3 acts to regulate cell differentiation and migration. There are limited data regarding the expression of MCT8 in the human fetal brain, however recent evidence suggests that both MCT8 and OATP1C1 are expressed in the blood-brain barrier (Roberts *et al.*, 2008). The psychomotor retardation that has been reported in males affected by MCT8 mutations suggests that MCT8 is critical for CNS development and it is postulated that this is associated with inadequate supply of THs to the developing brain (Biebermann *et al.*, 2005, Dumitrescu *et al.*, 2004, Friesema *et al.*, 2004, Holden *et al.*, 2005, Maranduba *et al.*, 2006, Schwartz *et al.*, 2005).

Deiodinase enzymes are also thought to play a role in TH availability to the fetal brain. Previous studies of deiodinases in human fetal cerebral cortex during the first and second trimesters of pregnancy showed that whilst the mRNA expression of D1 was

variable across gestation and D1 activity was undetectable, D2 mRNA and activity were present from 7-8 weeks of gestation and they peaked at 15-16 weeks (Chan *et al.*, 2002). D3 mRNA expression and enzymatic activity were detected from early first trimester. The D3 activity detected in human fetal cortex was significantly greater than that in adult cortex, indicating the importance of this mechanism for the local regulation of T_3 levels to protect the developing fetal brain from excessive T_3 supply. Kester *et al.* compared deiodinase activity in fetal brain samples from terminations of pregnancy (13 – 20 weeks of gestation) with neonatal brain samples from premature infants that died postnatally and they found that whilst D1 activity was undetectable, there was considerable D2 activity in the fetal cortex, which correlated positively with T_4 levels (Kester *et al.*, 2004). D3 activity was found in the cerebellum, midbrain, basal ganglia, brain stem, spinal cord and hippocampus, whilst it was lower in the cerebral cortex, choroid plexus and germinal eminence regions of the brain. D3 activity decreased after midgestation and was inversely correlated with T_3 levels in these tissues. These results suggest that the human fetal brain has the capacity to locally regulate the availability of T_3 via expressing deiodinase enzymes. The spatial and temporal changes in the expression of deiodinases might protect brain regions from excessive T_3 early in gestation.

TH action within the developing human brain has been demonstrated by the presence of nuclear binding sites for T_3 from week 10 of gestation with binding increasing with gestational age (Bernal and Pekonen, 1984). In a subsequent study, the mRNA expression of TR isoforms, TRα1, TRα2 and TRβ1, in first trimester fetal brains was demonstrated from as early as week 8 of gestation (Iskaros *et al.*, 2000). Our group has reported the presence of TRα1, TRα2, TRβ1 and TRβ2 mRNA in the fetal brain from week 7 of human pregnancy (Chan *et al.*, 2002, Kilby *et al.*, 2000). The protein

expression of all TR isoforms was most predominant in the pyramidal neurons of the cerebral cortex and the Purkinje cells of the cerebellum, indicating that these cell types are probably the targets of TH action within the fetal brain.

1.3.3   Importance of THs for placental development

A well functioning placenta is essential for normal fetal development, which is dependent on the placental-mediated transport of nutrients from the maternal to the fetal circulation and on the release of endocrine factors from the placenta (Malassine *et al.*, 2003). There is evidence that in addition to facilitating transplacental TH transport, the human placenta is a target organ for TH action and it has been postulated that the placenta is sensitive to the actions of TH for the regulation of its metabolism, differentiation and development. This hypothesis is supported by the association of maternal hyperthyroidism and hypothyroidism with pregnancy complications, including reduced birth weight, IUGR, pre-eclampsia, placental abruption, preterm delivery and spontaneous abortion (Davis, 1998; Idris, 2005; Casey, 2005; Blazer, 2003; Luewan, 2010; Pillar, 2010; Mestman, 2004). Studies using hypothyroid animal models have provided further evidence for the importance of THs in placental development. In rats, maternal hypothyroidism resulted in impaired reproduction and IUGR (Kumar and Chaudhuri, 1989). In similar studies by Pickard *et al.*, although maternal hypothyroxinaemia did not consistently affect rat placental development, it resulted in reduced cytosolic protein concentration at E15.5 and reduced total placental protein concentration at E19.5 with no effect on placental weight (Pickard *et al.*, 1993).

The hypothesis that THs affect trophoblast behaviour was subsequently confirmed by *in vitro* studies. Maruo *et al* investigated the effect of T_3 and T_4 treatment on

trophoblast behaviour using explants from early pregnancy and term placentae. Treatment with T_4 or T_3 caused an increase in the secretion of progesterone, estradiol-17β, hCGα, hCGβ and hPL by early placenta explants. In contrast, term placenta explants did not respond to TH treatment with increased endocrine activity, indicating that TH may play a role in the regulation of trophoblast differentiation and endocrine activity in early but not late pregnancy (Maruo *et al.*, 1991). Treatment of EVTs isolated from first trimester placentae with T_3 suppressed apoptosis via downregulating the expression of Fas and Fas ligand (Laoag-Fernandez *et al.*, 2004) and promoted invasion by EVTs (Oki *et al.*, 2004). These data suggest that the optimal level of TH may be essential in regulating endocrine activity, trophoblast differentiation and EVT invasion in early pregnancy.

It has also been suggested that TH acts in synergy with epidermal growth factor (EGF) to regulate placental growth and function. Treatment of first trimester placental explants with T_3 resulted in increased EGF secretion (Maruo *et al.*, 1995, Matsuo *et al.*, 1993). On the other hand, EGF increased D2 transcription and activity in the choriocarcinoma cell line, JEG-3, indicating that EGF has the potential to regulate T_4 conversion to T_3 by D2. Our group investigated the synergistic effects of EGF and T_3 on the function of CTs isolated from term placenta, JEG-3s and the EVT-like cell line, SGHPL-4. Treatment with either T_3 or EGF or co-treatment with T_3 and EGF promoted cell survival and proliferation of CTs and JEG-3s. In contrast, in SGHPL-4s all treatments attenuated cell proliferation. EGF treatment increased invasion and motility of SGHPL-4s, whilst co-treatment with T_3 attenuated invasion and enhanced cell motility (Barber *et al.*, 2005). These results indicate that THs affect trophoblast behaviour both directly and indirectly via interaction with other factors, such as EGF.

As mentioned in section 1.3.1 the human placenta expresses TH transporters that facilitate TH uptake by trophoblasts, deiodinase enzymes that locally regulate the availability of the different TH forms and TRs that mediate the genomic effects of T_3. There is currently limited information on the localisation of TH transporters in the placenta. The data available have demonstrated the presence of MCT8 in ST and CT cells throughout gestation. Furthermore, MCT8 protein was localised in interstitial and endovascular EVTs in early second trimester placental bed biopsies (Chan *et al.*, 2006). Therefore, MCT8 could facilitate TH transport in all these cell types. LAT1 and OATP4A1 are preferentially located at the maternal-facing side of ST in term placentae and may thus have an important role in the uptake of THs from the maternal circulation, however no information is available to date on their expression in early pregnancy (Okamoto *et al.*, 2002, Sato *et al.*, 2003). The localisation of MCT10, LAT2 and OATP1A2 transporters in the human placenta has not been investigated to date. Further studies are necessary to determine the contribution of these TH transporters to TH transport in specific placental cells.

Deiodinases 2 and 3 are present in the placenta throughout gestation and could locally modulate TH action. Immunohistochemistry has revealed that D2 and D3 proteins are present in both the CT and ST, with highest D2 expression in the CT and highest D3 expression in the ST (Chan *et al.*, 2003). Therefore, T_3 action may be more important in the CT, where it may regulate cell differentiation. It has been shown that placental tissue has a high nuclear binding capacity for T_3 (Ashitaka *et al.*, 1988, Banovac *et al.*, 1986). Our own study has demonstrated the expression of TRα1, TRα2 and TRβ1 within the nuclei of CTs, STs and stromal placental cells throughout gestation (Kilby *et al.*, 1998). TRα1, TRα2 and TRβ1 are also found in EVTs, as demonstrated by

immunohistochemistry in first and second trimester placental bed biopsies and in expression studies in primary cultures of first trimester EVTs (Barber *et al.*, 2005, Laoag-Fernandez *et al.*, 2004). These data indicate that the placenta expresses all the molecular apparatus required for TH action and metabolism. Further studies are required to better understand how these mechanisms regulate TH action within different placental cell types.

1.3.4   Abnormal maternal TH status and pregnancy complications

As discussed in section 1.1.2, IUGR is associated with increased risk of perinatal and neonatal mortality and morbidity and with life-long risk for cardiovascular disorder and diabetes in adult life. Thyroid status is one of several factors that have been postulated to play a critical role in the pathogenesis of IUGR. Maternal hyperthyroidism and overt and even subclinical maternal hypothyroidism have been associated with adverse pregnancy outcomes. There is increasing evidence that overt maternal hyperthyroidism is a risk factor for preterm delivery, low birth weight and IUGR (Luewan *et al.*, 2010, Mestman, 2004, Pillar *et al.*, 2010). Maternal hypothyroidism has been linked with increased incidence of placental abruption, preterm delivery and low birth weight (Davis *et al.*, 1988). In cases of subclinical hypothyroidism that are characterised by elevated concentration of serum TSH but normal serum T_4 concentration, even though the risk for low birth weight is lower compared with overt hypothyroidism, it is still elevated in comparison with the general population (Abalovich *et al.*, 2002, Alvarez-Pedrerol *et al.*, 2009, Casey *et al.*, 2005, Davis *et al.*, 1988, Idris *et al.*, 2005). Blazer *et al.* assessed the pregnancy outcome in cases where women were treated for hypothyroidism and found that birth weight was reduced in these pregnancies, indicating that close monitoring is required throughout pregnancy to ensure that treatment is adequate (Blazer *et al.*, 2003).

The above evidence has prompted further investigations of TH status in fetuses affected by severe, early onset IUGR. Using percutaneous, *in-utero* fetal blood sampling our group has determined that the serum concentrations of free T_4 and free T_3 are lower in fetuses affected by IUGR compared with matched controls, while there is no difference in serum TSH levels (Kilby *et al.*, 1998). As discussed in section 1.3.2, fetal development is sensitive to THs, thus changes in the concentration of circulating THs in fetuses affected by IUGR may contribute to the increased risk of adverse pregnancy outcomes associated with IUGR. The THs that are present in the fetal circulation at term originate from both fetal TH production and from transplacental transport from the maternal circulation. Therefore, reduced concentration of free T_4 and free T_3 in the fetal circulation with IUGR may be a result of either inefficient transplacental transport of maternal THs to the fetal circulation or inadequate production of THs from the fetal thyroid. Furthermore, changes in the metabolism of THs by deiodinase enzymes during their passage through the placenta or in fetal tissues, could also account for the altered concentrations of free T_4 and free T_3 in the fetal circulation.

Studies in placentae from normal pregnancies and pregnancies complicated by severe IUGR indicate that both TH action within the placenta and transplacental TH transport may be altered with IUGR. Even though there were no differences at the mRNA level, the protein expression of TRα1, TRα2 and TRβ1 was increased in IUGR placentae (Kilby *et al.*, 1998). It is postulated that changes in the expression of TRs may alter TH action within trophoblasts, and thus affect trophoblast function, which as discussed in the previous section is responsive to THs. Altered TH action may thus contribute to the reduced placental weight and altered placental morphology observed in IUGR pregnancies (see section 1.1.2). Furthermore, increased T_3 binding to TRs within the

nucleus of trophoblast cells may decrease the amount of T_3 available for efflux to the fetal circulation and thus contribute to the lower TH circulating levels in IUGR fetuses.

Comparisons of the mRNA expression and activity of the deiodinase enzymes D2 and D3 in human placentae from IUGR and normal pregnancies of similar gestational ages revealed no significant differences (Chan *et al.*, 2003). Furthermore, treatment of CTs isolated from normal term placentae with 10 nM or less T_3 did not affect D2 and D3 mRNA expression. This evidence suggests that unlike other tissues, like fetal brain, where increased D2 activity may compensate for low TH levels (Bianco *et al.*, 2002), the placenta cannot compensate for decreased THs in the fetal circulation by modulating the expression of deiodinases. In contrast, the placental expression of the TH transporter MCT8 is increased in IUGR pregnancies compared with gestationally-matched controls and it is postulated that increased MCT8 expression in the IUGR placenta may be a compensatory mechanism for the reduced TH levels in the fetal circulation (Chan *et al.*, 2006). Better understanding of transplacental TH transport in normal and IUGR pregnancies requires the assessment of the expression of all the TH transporters expressed in the human placenta in normal and IUGR pregnancies. This information would help to assess the possibility that changes in TH transporter expression in the placenta may contribute to the reduced TH levels in the circulation of fetuses affected by IUGR.

1.4   Monocarboxylate transporter 8

Transplacental transport of THs is important for both fetal and placental development. However, there is limited information about the mechanisms that may regulate TH transport in the human placenta, including the expression of TH transporters. Of all the TH transporters reported to date, MCT8 has received special interest following reports of mutations of the MCT8 gene that are associated with X-linked severe neurodevelopmental disorders in affected males, suggesting that MCT8 has an essential role in the developing fetal brain.

1.4.1   Expression

The gene encoding human MCT8 is located on the X-chromosome (Xq13.2) and is subject to X-chromosome inactivation (Lafreniere *et al.*, 1994). The human MCT8 mRNA contains two alternative translation start sites at positions 167 and 389 resulting at the production of a protein of either 613 or 659 amino acids (Friesema *et al.*, 2006, Lafreniere *et al.*, 1994). Kinne *et al.* have shown that human cell lines express both transcripts *in vitro* (Kinne *et al.*, 2009). In contrast, rodent species express the shorter form of the MCT8 protein (Friesema *et al.*, 2006). The functional characteristics of the short and long forms of human MCT8 protein have not been compared to date.

MCT8 is widely expressed. Lafreniere *et al.* detected MCT8 mRNA in human brain, liver, placenta, heart, lung and kidneys (Lafreniere *et al.*, 1994). The expression of MCT8 mRNA and protein in the brain was confirmed by Roberts *et al.*, who demonstrated that MCT8 is expressed in cerebral microvessels in human, mouse and rat, indicating that MCT8 may play a role in the transport of THs across the blood-brain

barrier (Roberts *et al.*, 2008). Furthermore, the expression of MCT8 mRNA and protein in the human placenta throughout gestation suggests that MCT8 contributes to transplacental TH transport (Chan *et al.*, 2006). Friesema *et al.* demonstrated the expression of MCT8 protein in rat brain, liver, kidney and heart (Friesema *et al.*, 2003) and Capelo *et al.* demonstrated the expression of MCT8 mRNA in femurs of mouse fetuses both prenatally and postnatally, suggesting a role for MCT8 during bone development (Capelo *et al.*, 2009).

1.4.2   TH transport characteristics

The ability of MCT8 for uptake of THs was first demonstrated by transport studies in *Xenopus* oocytes transfected with rat MCT8 and the calculated Km values were 4 μM for T_3, 4.7 μM for T_4, and 2.2 μM for rT_3 (Friesema *et al.*, 2003). Furthermore, it was shown that MCT8 does not transport the sulphated forms of T_4. In a subsequent study, the Km value of human MCT8 transfected in MDCK1 cells for T_3 was calculated as 0.86 μM (Kinne *et al.*, 2009). Although the two studies were performed in different systems, these results indicate that human MCT8 is a more potent transporter for T_3 than rat MCT8. The study by Friesema *et al.* also demonstrated that transport of T_4 was strongly inhibited by L-T_4, D-T_4, L-T_3, D-T_3, Triac, BrAcT_3 and BSP, whilst T_3 uptake was less affected by those molecules (Friesema *et al.*, 2003). Transport of T_3 and T_4 were temperature-dependent and whilst T_3 transport was Na^+-independent, T_4 transport was slightly inhibited in the absence of Na^+. The ability of MCT8 to transport amino acids or monocarboxylates was tested using leucine, phenylalanine, tryptophan, tyrosine and lactate and it was shown that MCT8 does not transport these compounds. The function of human MCT8 as a TH transporter was further confirmed by transport studies in COS1 and JEG-3 cells transfected with human MCT8 (Friesema *et al.*, 2006). In a subsequent study,

it was shown that in addition to mediating uptake of THs, MCT8 also facilitates TH efflux (Friesema *et al.*, 2008).

1.4.3   Mutations

Mutations of the MCT8 gene have been reported in over 30 unrelated families around the world (**Figure 1-10**). Friesema *et al.* and Dumitrescu *et al.* first identified MCT8 mutations in the genome of males affected by severe neurological and developmental disorder (Dumitrescu *et al.*, 2004, Friesema *et al.*, 2004). The affected patients displayed severe mental retardation, lack of speech, rudimentary communication skills, feeding problems and inability to sit, crawl or stand. The patients also demonstrated altered thyroid status with low total and free T_4 and high total and free T_3, whilst the concentration of TSH ranged from normal to slightly elevated. Due to the location of the MCT8 gene on the X chromosome, males that inherit the mutation from their mothers that are heterozygous for the mutation display a severe phenotype. In contrast, heterozygous female carriers of the mutation in these families had a milder thyroid phenotype with low to normal total and free T_4, normal to high total and free T_3 and normal TSH and displayed no neurodevelopmental abnormalities. Several different mutations of the MCT8 gene were subsequently reported in male patients affected by neurodevelopmental abnormalities including paroxysmal kinesigenic dyskinesias and Allan-Herndon-Dudley syndrome (Biebermann *et al.*, 2005, Brockmann *et al.*, 2005, Frints *et al.*, 2008, Fuchs *et al.*, 2009, Holden *et al.*, 2005, Jansen *et al.*, 2007, Jansen *et al.*, 2008, Kinne *et al.*, 2009, Maranduba *et al.*, 2006, Namba *et al.*, 2008, Schwartz *et al.*, 2005, Visser *et al.*, 2009). Furthermore, Frints *et al.* identified a female patient affected by Allan-Herndon-Dudley syndrome due to a chromosomal translocation affecting the MCT8 gene accompanied by

non-random inactivation of the normal X-chromosome that resulted in loss of MCT8

expression (Frints *et al.*, 2008).
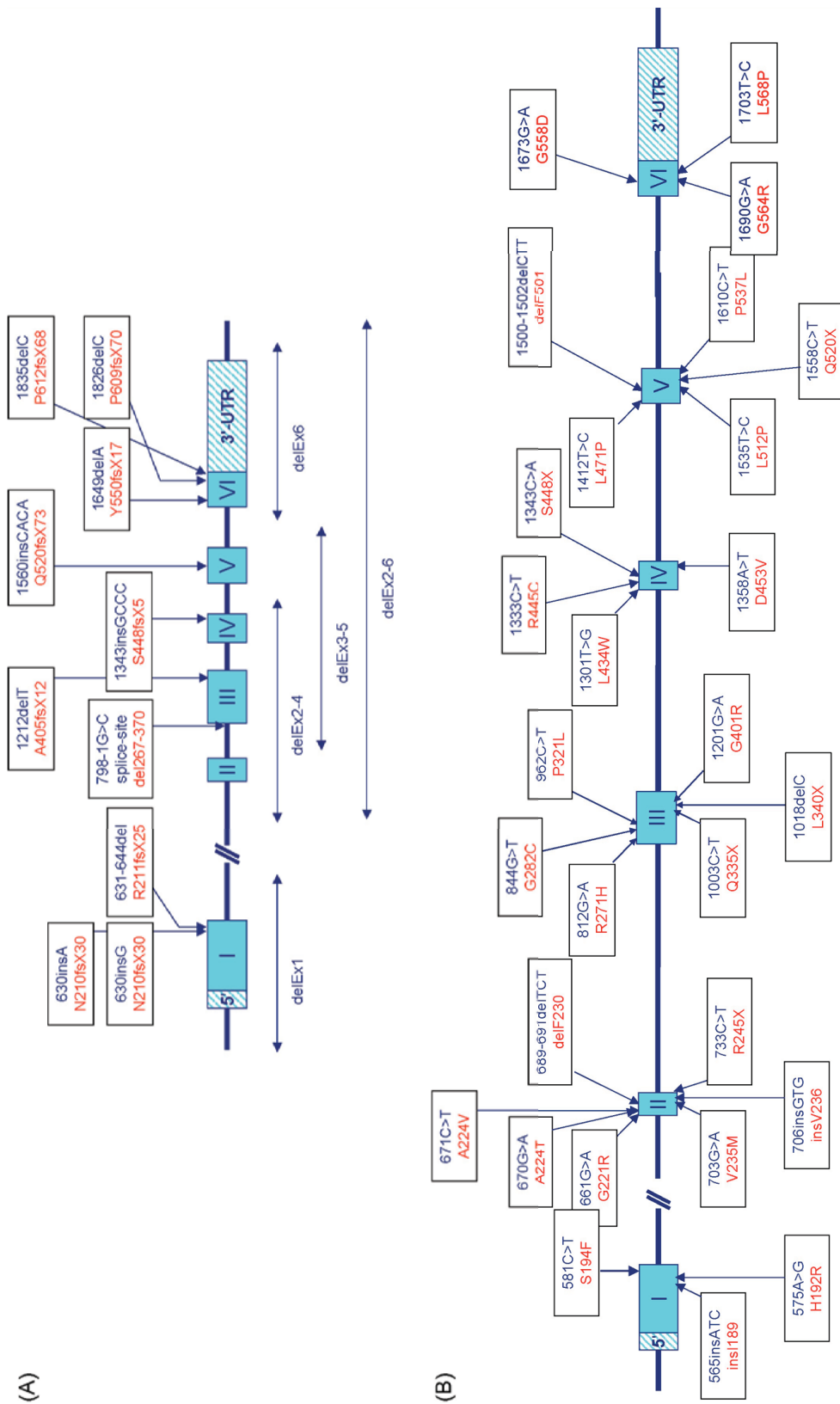
Figure 1-10: *MCT8 mutations. The genomic strucutre of the MCT8 gene is shown and mutation identifed are annotated. (**A**) Large deletions and frame shifts. (**B**) Triplet deletions, insertions, missense and nonsense mutations. Figure from (Friesema et al., 2010).*

1.4.4   Functional characteristics of wild-type and mutant MCT8 proteins

The identification of several mutations in male patients displaying neurodevelopmental delay revealed variations in the severity of the phenotype. Whilst some mutations (L512P, V235M, S448X, insI189, delF230) resulted in inability to stand, walk or speak, patients affected by other mutations (L434W, L568P, S194F) could walk independently and in some cases developed some dysarthric speech (Dumitrescu *et al.*, 2004, Jansen *et al.*, 2008). This prompted the investigation of the functional characteristics of different MCT8 mutants. Of the MCT8 mutants tested so far A150V, A224V, L471P, R245X, delF230, V235M, S448X and insI189 demonstrated no residual T_3 transport when transfected in MCT8-null cell lines, whilst mutants 1834delC, R271H, L434W, L568P and S194F demonstrated residual T_3 transport (Biebermann *et al.*, 2005, James *et al.*, 2009, Jansen *et al.*, 2007, Jansen *et al.*, 2008, Maranduba *et al.*, 2006). Immunohistochemistry revealed changes in the subcellular localisation of the different mutants. Wild-type MCT8 protein was found predominantly at the plasma membrane consistent with its transporter function. However, mutants A150V, V235M, insI189, delF230 and L471P were instead retained in the cytoplasm (Biebermann *et al.*, 2005, James *et al.*, 2009, Jansen *et al.*, 2008). Mutants L434W, L568P and S194F were found predominantly in the cytoplasm but were also detected in the plasma membrane (Jansen *et al.*, 2008). Protein stability was also found to differ between different mutated forms of MCT8. When transfected in MCT8-null cell lines, mutants A224V, R271H, L434W and S194F were highly expressed, whilst mutants L471P, delF230, R245X, V235M, L568P and insI189 showed little protein expression and S448X was undetectable (Jansen *et al.*, 2007, Jansen *et al.*, 2008). These results demonstrate that the MCT8 mutations tested result in complete or partial loss of MCT8 transport function, which is often associated

with impaired protein stability and impaired localisation of the mutant protein to the plasma membrane. Furthermore, there is a correlation between the functional characteristics of mutated MCT8 proteins and the severity of the phenotype in patients affected by these mutations. In a subsequent study, Kinne *et al.* transfected MCT8 mutants in different cell lines and reported that the protein stability and membrane translocation of MCT8 mutants was different depending on the cell line they were expressed in, thus raising the possibility that the function of MCT8 mutants *in vivo* may differ in different cell types (Kinne *et al.*, 2009).

Further *in vitro* studies have assessed the function of MCT8 in neuronal cells. Sugiura *et al.* assessed the role of MCT8 in the differentiation of mouse embryonic stem cells (ES) to neural progenitors and they demonstrated that the expression of MCT8 increased with differentiation (Sugiura *et al.*, 2007). Furthermore, when MCT8 was transfected in ES cells, the expression of neural stem cell markers, SOX-1 and MAP-2 was sustained for longer compared with cells transfected with an empty vector. However, even though TH accelerates the differentiation of neural stem cells to oligodendrocytes, transfection with MCT8 did not produce a similar effect. James *et al.* assessed the role of MCT8 in N-Tera2 cells, a cell line that has characteristics of central nervous system precursors and found that even though transfection with wild-type MCT8 did not affect apoptosis or ER stress, it reduced cell proliferation, whilst the L471P MCT8 mutant had no effect on cell number (James *et al.*, 2009). Interestingly, this effect was demonstrated both in the absence and in the presence of T_3 and it was confirmed in the choriocarcinoma cell line JEG-3 that has no endogenous expression of MCT8 or functional TRs. These results demonstrate that MCT8 affects cell proliferation via T_3-independent mechanisms and suggest that MCT8 has an additional role apart from TH transport.

There is limited information concerning the regulation of MCT8 expression. Capelo *et al*. demonstrated that MCT8 mRNA in the osteoblastic cell line, MC3T3-E1, decreased following treatment with T_3, suggesting that the expression of MCT8 may be regulated to maintain optimal concentration of intracellular T_3 (Capelo *et al.*, 2009). However, this observation has not been confirmed in other cell types to date. MCT8 contains a PEST domain at the N-terminus. PEST domains are associated with rapid protein degradation (Rogers *et al.*, 1986). However, *in vitro* studies suggest that although MCT8 protein is a target for the ubiquitin-proteasome pathway, it is not subject to rapid degradation (Jansen, 2008).

Other members of the MCT family function as heterodimers with ancillary proteins, such as embigin, basigin and neuroplastin. Even though MCT8 does not associate with these proteins, transfected MCT8 forms homodimers and tetramers that are detected at 120 kDa, 250 kDa by Western immunoblotting (Visser *et al.*, 2009). Biebermann *et al*. demonstrated the ability of both wild-type MCT8 and of MCT8 A150V mutant to form homodimers and to dimerise with each other, indicating that the A150V mutation does not affect the ability of MCT8 to form dimers (Biebermann *et al.*, 2005). Visser *et al*. subsequently showed that deletion of the transmembrane domains 4-6 or individual mutations of cysteine residues to alanine do not affect MCT8 homodimerisation (Visser *et al.*, 2009). The occurrence of MCT8 homodimers has not been demonstrated in cells or tissues expressing endogenous MCT8 to date. Furthermore, the implications of MCT8 homodimerisation on transport function have not been investigated.

1.4.5   MCT8 knockout mice

The clinical observations in male patients with MCT8 mutations demonstrate the importance of MCT8 for the development of the CNS. In the mouse, the expression of MCT8 mRNA was localised in different brain regions by *in situ* hybridisation including the cerebral cortex, basal ganglia, amygdala, olfactory bulb, hippocampus, hypothalamus, choroid plexus, median eminence and cerebellum (Heuer *et al.*, 2005). There are conflicting reports on the expression of MCT8 at the blood-brain barrier, where it could play a role in the supply of THs to the brain. Whilst Roberts *et al.* detected MCT8 mRNA and protein in cerebral microvessels in human, rat and mouse, Heuer *et al.* detected only limited expression of MCT8 mRNA in blood vessels in sections from mouse brain (Heuer *et al.*, 2005, Roberts *et al.*, 2008). The detection of MCT8 in murine neurons led to the hypothesis that MCT8 has a role in the uptake of T_3 by neurons (Heuer *et al.*, 2005, Wirth *et al.*, 2009). Dumitrescu *et al.* and Trajkovic *et al.* independently generated MCT8 knockout mouse models to further determine the role of MCT8 in brain development (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007).

In contrast to the adverse neurodevelopmental phenotype observed in human patients affected by MCT8 mutations that result in loss of MCT8 function, MCT8 knock-out mice developed normally and were indistinguishable from their wild-type or heterozygous littermates with no differences in postnatal growth rate, brain weight, fertility or reproduction (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007). However, similar to patients affected by MCT8 mutations, MCT8 knockout mice had altered TH status with decreased serum T_4 and increased T_3 (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007). Investigation of TH status in different organs revealed that whilst the brain of MCT8-null animals was in a hypothyroid state, the liver and kidneys of these animals

were in a hyperthyroid state (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007, Trajkovic-Arsic *et al.*, 2009). Furthermore, injection with $[^{125}I]$-T_4 or $[^{125}I]$-T_3 demonstrated that whilst accumulation of T_4 in the brain of MCT8-null mice was not altered, T_3 accumulation was severely impaired. This was in contrast to observations in the liver where there was no effect on intracellular accumulation of THs (Trajkovic *et al.*, 2007). D2 activity was upregulated and D3 activity was downregulated in the CNS of MCT8-null mice possibly reflecting a compensatory mechanism in response to the low concentration of THs. However, these changes they were not sufficient to restore normal T_3 availability (Trajkovic *et al.*, 2007). These observations suggest that whereas MCT8 may not have an important role in TH transport in the liver, it is essential in facilitating T_3 entry in the murine brain. In addition, the lack of change in the level of TSH in spite of the increased serum T_3 in MCT8-null mice suggests that the pituitary is resistant to T_3 possibly due to the impaired T_3 entry to the brain (Trajkovic *et al.*, 2007).

The difference in brain development between mice and humans could explain the absence of a neurodevelopmental phenotype in MCT8-null mice. In addition, other TH transporters may compensate for the lack of MCT8 in mice. Wirth *et al.* performed a detailed immunohistochemical neurological and behavioural screen of MCT8-deficient mice and reported that knockout of MCT8 expression does not alter brain histogenesis and is not associated with differences in reflexes, locomotion, grip strength or movement co-ordination (Wirth *et al.*, 2009). However, MCT8-null mice developed decreased anxiety-related behaviour, hyperalgesia-related behaviour and increased latency to grooming. T_3 uptake studies in cultures of neurons from MCT8 knockout and wild-type mice demonstrated that even thought T_3 uptake was significantly decreased in MCT8-null neurons, it was not completely diminished suggesting the expression of TH transporters in

addition to MCT8 in mouse neurons. The authors assessed the expression of MCT10, OATPs and LATs and they reported that LAT2 is co-expressed with MCT8 in mouse neurons, but not in human developing neurons (Wirth *et al.*, 2009). Therefore, in mice, LAT2-mediated transport of T_3 to developing neurons may partially compensate for the absence of MCT8 and may partly explain the absence of a severe neurological phenotype in MCT8-deficient mice. Furthermore, there is evidence that OATP1C1 is expressed in mouse but not human cerebral microvessels suggesting that it could compensate for the lack of MCT8 in MCT8-deficient mice (Roberts *et al.*, 2008).

1.4.6   Implications for transplacental TH transport

As discussed in section 1.3.1, MCT8 is expressed in the placenta from week 6 of gestation, suggesting an important role for the transport of maternal THs to the developing fetus throughout gestation (Chan *et al.*, 2006). Furthermore, the expression of MCT8 is increased in human placentae from pregnancies complicated by severe IUGR. Assessment of the functional role of MCT8 in the human placenta is necessary to elucidate its importance for transplacental TH transport and its effects on placental cells. Even though the intrauterine growth, as assessed by birth weights, of fetuses affected by MCT8 mutations is normal (Fuchs *et al.*, 2009, Namba *et al.*, 2008, Schwartz *et al.*, 2005), the possibility that transplacental transport of THs is altered in these pregnancies cannot be excluded. Such alterations could further affect fetal serum levels of THs and the development and maturation of the CNS and other organs.

1.5   Aims and hypothesis

The importance of maternal THs for fetal development is well established. However, the mechanisms that regulate transplacental transport of maternal THs in normal pregnancies and in pregnancies complicated by IUGR are not well understood. Furthermore, there is increasing evidence that THs affect placental development. Therefore, TH transporters are likely to play an important role in regulating the supply of maternal THs to the fetal circulation as well as to placental cells.

We hypothesise that the co-ordination of the expression of TH transporters in the placenta throughout gestation is important for the regulation of transplacental transport of maternal THs and that changes in the placental expression of TH transporters in pregnancies complicated by IUGR may affect the supply of maternal THs to the fetal circulation. The reports that there is altered expression of the TH transporter MCT8 and of TRs in placentae from IUGR pregnancies led to the hypothesis that the effects of T_3 on trophoblast cells may differ between normal and IUGR pregnancies. Furthermore, we hypothesise that the TH transporter MCT8 is likely to have an important role in placental function through the facilitation of the effects of THs on trophoblast cells.

The aims of this thesis were:

•   To describe the expression of TH transporters in the human placenta throughout gestation and compare their expression between uncomplicated pregnancies and pregnancies complicated by IUGR.

•   To assess the impact of TH on CT cells from normal and IUGR pregnancies and to compare TH transport by these cells.

- To investigate the function of the TH transporter MCT8 in placental cells *in vitro* using invasive EVT-like cells and primary cultures of CTs from term placentae.

- To assess the role of MCT8 in placental development and fetal growth *in vivo* using the MCT8 knockout mouse model.

Chapter 2: Expression of Thyroid Hormone Transporters in the Human Placenta across Gestation and Changes with Intrauterine Growth Restriction

2.1 Introduction

It is well known that THs are essential for normal fetal growth and development, particularly for the development of the CNS (Haddow *et al.*, 1999, Pop *et al.*, 2003, Pop *et al.*, 1999). Maternal THs are present in human embryonic cavities since the first trimester of pregnancy (Calvo *et al.*, 2002, Calvo *et al.*, 1990, Contempre *et al.*, 1993, Thorpe-Beeston *et al.*, 1991, Vulsma *et al.*, 1989). Before the onset of fetal TH production at week 14 of human pregnancy, maternal THs are the only source of THs in the fetal circulation (Morreale de Escobar *et al.*, 2004). There is ongoing fetal demand for maternal TH supply throughout pregnancy and it is estimated that maternal T_4 comprises 30% of the total circulating T_4 present in the fetus at term (Chan *et al.*, 2009).

In addition, it is believed that THs are important for placental development. The human placenta expresses the TR isoforms, TRα1, TRα2 and TRβ1, indicating that it is a target organ for TH action (Kilby *et al.*, 2005). *In vitro*, THs promote the invasive capacity of primary EVTs and EVT-like cell lines and down-regulate apoptosis in primary EVTs (Barber *et al.*, 2005, Canettieri *et al.*, 2008, Laoag-Fernandez *et al.*, 2004, Maruo *et al.*, 1991, Maruo *et al.*, 1995, Matsuo *et al.*, 1993, Oki *et al.*, 2004). THs may therefore play a role in the regulation of trophoblast invasion and maternal spiral artery remodelling. Furthermore, THs increase the endocrine activity of explants from first trimester placentae (Maruo *et al.*, 1991).

Abnormal maternal TH status has been linked with increased risk for pregnancy complications associated with malplacentation, such as IUGR (Blazer *et al.*, 2003, Casey *et al.*, 2005, Davis *et al.*, 1988, Idris *et al.*, 2005, LaFranchi *et al.*, 2005, Luewan *et al.*, 2010, Mestman, 2004, Pillar *et al.*, 2010). In IUGR fetuses, the serum levels of free T_3

and free T_4 are reduced (Kilby *et al.*, 1998). Furthermore, there is increased mRNA and protein expression of the TH transporter MCT8 in biopsies from IUGR placentae compared with gestationally matched controls (Chan *et al.*, 2006). These findings suggest that transplacental TH transport may be altered in IUGR pregnancies.

Therefore, there is a necessity for an efficient TH transport system in the placenta to facilitate the uptake of THs from the maternal circulation and their efflux into the fetal circulation throughout pregnancy. Indeed, a range of TH transporters have been identified in the human placenta to date. These are MCT8 (Chan *et al.*, 2006), MCT10 (Park *et al.*, 2005), LAT1 (Okamoto *et al.*, 2002), LAT2 (Kudo and Boyd, 2001), OATP1A2 (Patel *et al.*, 2003) and OATP4A1 (Briz *et al.*, 2003, Patel *et al.*, 2003, Sato *et al.*, 2003). However, there is limited information about the expression of these transporters in the placenta throughout gestation. This study aims to describe the ontogeny of these TH transporters in a large cohort of placental samples ranging from week six of pregnancy until term and to compare their expression between samples from IUGR pregnancies and gestationally matched controls.

2.2   Materials and Methods

All chemicals used in this thesis were obtained from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

2.2.1   Tissues

This study had approval of the Local Research Ethics Committee (South Birmingham) and the Research and Development Committee of the Birmingham Women's Hospital.

Placental biopsies were collected from 110 normal placental samples. The samples were grouped as follows. The first group was from 6 to 10 weeks of gestation, which is before the oxygen level in the placenta increases (n=39). The second group ranged from 11 to 14 weeks of gestation, which is before the onset of TH production by the fetus (n=18). The third group was from 15 to 20 weeks (n=4). Samples from 27 to 34 weeks are from cases that had to be delivered at the early third trimester (n=9). The last group consisted of samples obtained at 37 to 41 weeks of pregnancy, for cases that were delivered at term (n=40). Samples from 6 to 20 weeks were collected following surgical termination of pregnancy for reasons other than fetal abnormality (in accordance with the Polkinghorne report and the Human Tissue Act). The gestational ages of the pregnancies were determined by ultrasound scan in the first trimester of pregnancy. The normal early third trimester group (27-34 weeks) consisted of samples obtained from placentae of appropriately grown for gestational age fetuses, which were collected following emergency caesarean section without labouring for placenta praevia (n=3), maternal tumours (n=3) or prelabour rupture of membranes (with no clinical evidence of infection)

with a breech presentation (n=3). The normal late third trimester group (37-41 weeks) consisted of placentae from normal pregnancies delivered by elective caesarean section for previous caesarean section, breech presentation or patient choice.

Placental biopsies were also collected from 22 cases of severe IUGR in the absence of maternal hypertension. These were diagnosed prospectively using ultrasound and they had at least three of the following four characteristics: (i) ultrasound measurement of the fetal abdominal circumference of less than or equal to the third centile for gestation, (ii) abdominal circumference growth velocity of less than 1.5 standard deviations over 14 days, (iii) severe oligohydramnios (amniotic fluid index ≤ tenth centile for gestation) and (iv) absent end diastolic flow velocity in the umbilical artery Doppler velocity waveform measurements. None of the babies had known chromosomal or structural abnormalities. These criteria selected a severe and relatively homogenous phenotype. IUGR placenta were collected at 25-32 weeks (early onset IUGR; n=17) and at 37-38 weeks (late onset IUGR; n=5); all delivered by caesarean section without labouring. The early onset IUGR group represents cases at the more severe end of the IUGR disease spectrum with a 53% neonatal mortality in this cohort.

Immediately after delivery of the placenta, multiple random biopsies were obtained by dissecting the placental tissue off the chorionic plate, ensuring that there was no residual membrane attached to the sample and washing in sterile saline solution (0.9%). The biopsies were snap-frozen in liquid nitrogen and stored at -80ºC until required for extraction of RNA or protein.

2.2.2   RNA extraction

Approximately 100 mg of placental tissue were homogenised in 1 ml TRI reagent (Ambion, Warrington, UK) and total RNA was extracted following the manufacturer's guidelines. Briefly, the homogenised samples were centrifuged for 10 minutes at 12,000 × g at 4°C and the supernatant was transferred to clean tubes. Chloroform (200 μl per ml of TRI reagent) was added. The solutions were mixed well, incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 × g at 4°C. The aqueous phase containing the RNA was transferred to clean eppendorfs and 500 μl of isopropanol were added per ml of TRI reagent. The solutions were mixed, incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 × g at 4°C. The supernatant was discarded and 1 ml of 75% ethanol was added to each pellet. Following vortexing, the samples were centrifuged for 5 minutes at 7,600 × g at 4°C, the supernatant was discarded and the pellet was left to air dry for approximately 10 minutes. The RNA pellet was dissolved in 100 μl of RNAse-free water and the samples were heated at 55°C for 15 minutes to dissolve the RNA pellet. The absorbance of the samples at 260 (A_{260}) and 280 nm (A_{280}) was measured using a spectrophotometer (Geneflow, UK). RNA purity was assessed by calculating the A_{260}/A_{280} ratio, which was consistently between 1.7 and 2. The RNA concentration of the samples was calculated using the Beer-Lambert law; an A_{260} of 1 corresponds to a RNA concentration of 40 μg/ml in a 1 cm pathlength. The RNA samples were stored at -80 °C.

2.2.3   Reverse Transcription

RNA was reverse transcribed using Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega, Madison WI, USA). Total RNA (1 μg) was made up to a total

volume of 9.75 μl using nuclease free water and it was incubated at 70°C for 10 minutes.

Then it was mixed with 5 mM MgCl$_2$, 1 × reverse transcription buffer (10 mM Tris-HCl;

pH 9.0; 50 mM KCl; 0.1% Triton® X-100), 0.5 μg of random hexamer primers, 4 mM of

deoxynucleotide triphosphate (dNTP) mix, 20 units of ribonuclease inhibitor (RNasin)

and 15 units of AMV reverse transcriptase in a total reaction volume of 20 μl. The

reactions were sequentially incubated at room temperature for 10 minutes, at 42°C for 60

minutes, at 95°C for 5 minutes and at 4°C for 5 minutes. The samples were diluted 1:1

with RNAse free water and were stored at -20°C.

2.2.4 Design and validation of primer and probe sets for quantitative TaqMan polymerase chain reaction (PCR)

Primers and probe for MCT8 had been previously designed and validated in our

laboratory (Chan *et al.*, 2006). Primers and probes for MCT10, LAT1, LAT2, CD98,

OATP1A2 and OATP4A1 were designed using Primer Express v2.0 software (Applied

Biosystems, Fostercity, CA, USA) (**Table 2-1**). All amplicons were crossing an exon-

exon boundary and each set was designed so that the sequence for either the probe or one

of the primers spanned two adjacent exons. Primers were synthesised by Alta Bioscience

(Birmingham, UK). TaqMan Probes were synthesised and modified with the fluorophore

FAM at the 5' end and the quencher TAMRA at the 3' end (Eurogentec, Seraing,

Belgium). Genomic amplification was excluded by comparing the amplification of cDNA

with control reverse transcriptase reactions without AMV.

The expression of the target gene was normalised to the expression of the

housekeeping gene encoding the small subunit ribosomal RNA, 18S. The potential

housekeeping genes 18S, TATA box-binding protein (TBP), succinate dehydrogenase

complex, subunit A (SDHA) and cyclophilin were previously tested in our laboratory on a subset of placental samples to assess for stable expression both across gestation and between normal and IUGR placentae. Normfinder, a model-based approach was used to identifying the most stable housekeeping genes (Andersen *et al.*, 2004) and 18S, TBP and SDHA were identified as the genes with the most stable expression, consistent with a report on quantitative RT-PCR analysis in human placenta (Patel *et al.*, 2003). 18S was amplified using a predeveloped TaqMan assay containing a VIC-labelled probe and primers for 18S (Applied Biosystems). Validation experiments were carried out to determine if duplex reactions could be performed with amplification of the target gene and of the 18S control in the same tube. Serial dilutions of cDNA from term placental biopsies ranging between 12.5 and 75 ng per reaction were amplified using primer and probe sets for 18S and for the target gene in a duplex reaction. The Ct values (the cycle number at which logarithmic PCR plots cross a threshold line) for 18S and for the target gene were plotted against the cDNA concentration and a linear regression line was fitted. A difference of <0.1 between the gradients of the regression lines indicated that there was no interference between the two reactions and the two cDNAs could be detected in duplex reactions.

Table 2-1: *Sequences of primers and probes for TaqMan PCR.*

Gene	Forward primer	Reverse primer	Probe	Amplicon length (bp)
MCT8 (NM_006517)	CAACGCACTTA CCGCATCTG	GTAGCCCCAATA CACACCAAGAG	TCCACATACTTCAT CAGGTGTACATAG GGAACAAA	150
MCT10 (NM_018593)	GATTCATGTCTA TACCCATGACT GTTG	CACATCATAGGA GCCCAGTTTGT	CCCACCCATTGCAG GGTTACTTCGT	77
LAT1 (NM_003486)	AAATGATCAAC CCCTACAGAAA CCT	ACGTACACCAGC GTCACGAT	CCTGGCCATCATCA TCTCCCTGCC	73
LAT2 (NM_012244)	AAATCTGGAGG TGACTACTCCTA TGTC	GATCACCAGCAC AGCAATCC	AGCCTCAGGAACC CAGCCAGTCCT	87
CD98 (NM_001012661)	TGAGATTGGCC TGGATGCA	GGACTCATCCCAC AGCATGAC	CCCTTCCTGGACAG CCTATGGAGGC	73
OATP1A2 (NM_134431)	GTGAACACAGA TGATCTGATCAT AACTC	AACTCCTGCACA AATCAGAAAGC	ATGCACCGACCCA ACGAGTGTCAGT	87
OATP4A1 (NM_016354)	GCAGCCACGGA GACGAA	AAAGATTCTGAG GGATACAGCTAC AGT	CTCGGTACACCTTC TGGCCGTCCA	70

All sequences are given in the 5'-3' direction. Underlined base pairs correspond to boundaries between two adjacent exons. Basepairs (bp).

2.2.5 Quantitative TaqMan PCR

TaqMan PCR was carried out using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). cDNA (1 μl) was mixed with 22.5 pmoles of forward primer, 22.5 pmoles of reverse primer, 3.75 pmoles TaqMan probe, 1.25 μl of 18S predeveloped TaqMan Assay (Applied Biosystems) and 12.5 μl qPCR Mastermix Plus QuickGold Star (Eurogentec) in a total volume of 25 μl. All reactions were performed in duplicate. The PCR conditions were as follows. The samples were incubated at 50°C for 2 minutes and then they were heated at 95°C for 10 minutes followed by 40 cycles of 15

seconds at 95°C and 1 minute at 60°C. Data were expressed as Ct values. Relative quantification of the expression of each gene was determined using the ΔCt method. For each sample fold changes in gene expression were calculated with the equation $2^{-\Delta Ct}$ (ΔCt = sample Ct − calibrator Ct). The calibrator was determined for each gene as the sample with the highest expression. For each sample, the expression of the target gene was normalised to 18S to correct for differences in the efficiency of the reverse transcription. The mean gene expression for the normal term placenta samples was assigned the arbitrary value of 1. The relative mRNA expression for each sample compared with the mean for term placenta was calculated and these values were used for statistical analysis.

2.2.6   Generation of MCT8 antisera

Rabbit polyclonal antisera for human MCT8 (Ab4790; Sigma-Genosys, Suffolk, UK) were raised against a synthetic polypeptide conjugated to keyhole limpet hemocyanine comprising amino acids 79-92 (SQASEEAKGPWQEA) at the intracellular N terminus of the MCT8 protein. The antisera from final bleeds were affinity purified and their specificity was confirmed by Western immunoblotting using antibody solutions that were pre-incubated with and without the immunising peptide on whole cell protein extracted from human placenta or from MCT8-null JEG-3 choriocarcinoma cells transfected with either human MCT8 or the empty plasmid vector (VO).

2.2.7   Protein extraction

Approximately 100 mg of placental tissue from randomly selected samples from each group were homogenised in 1 ml RIPA buffer (pH 7.4; 50 mM Tris-HCl, 150 mM NaCl, 1% v/v Igepal CA-630, 6 mM sodium deoxycholate, 1 mM EDTA) containing 60 µl/ml protease inhibitor cocktail (containing 4-(2-aminoethyl)benzenesulphonyl fluoride,

pepstatin A, E-64, bestatin, leupeptin and aprotinin). Protein concentration was determined using the colorimetric Bio-Rad Dc protein assay (Bio-Rad, Hertfordshire, UK). Protein standards of bovine serum albumin (BSA) in RIPA buffer were prepared at the following concentrations: 0, 0.25, 0.5, 0.75, 1 and 2 μg/μl. Equal amounts (5 μl) of each standard and sample were added to separate wells of a 96-well plate in duplicate followed by 25 μl of Reagent A and 200 μl of Reagent B. The samples were incubated at room temperature for 15 minutes and the absorbance at 690 nm was measured using a Victor[3] multilabel counter (Perkin Elmer, Waltham, MA, USA). The standard curve that was produced from the BSA standards was used to determine the protein concentration of the samples.

2.2.8   Western immunoblotting

Protein samples (30 μg) were mixed with equal volumes of Laemmli buffer (Bio-Rad) with dithiothreitol (DTT) at a final concentration of 175 mM. The samples were sonicated and incubated at room temperature for 1 hour. They were centrifuged at 13,000 rpm for 10 minutes and loaded on electrophoresis gels made up with 10% (w/v) acrylamide resolving gel (7 ml) and 5% (w/v) acrylamide stacking gel (2.5 ml). The resolving gel consisted of 8% (w/v) acrylamide 0.2% (w/v) bis-acrylamide, 0.375 M Tris HCl, 3.5 mM sodium dodecyl sulphate (SDS), 4.4 mM ammonium persulphate (APS) and 0.1% (v/v) N,N,N′,N′-Tetramethylethylenediamine (TEMED). The stacking gel consisted of 5% (w/v) acrylamide, 0.13% (w/v) bis-acrylamide, 0.2 M Tris HCl, 6.9 mM SDS, 8.7 mM APS and 0.2% (v/v) TEMED. The gels were immersed in 1× running buffer (pH 8.3) containing 25 mM Tris base, 192 mM Glycine and 3.5 mM SDS in distilled water. The samples and 6 μl of protein standards (Dual Colour Marker; Bio-Rad) were allowed to migrate through the gels by electrophoresis at 70 volts for approximately half an hour and

then at 100 volts for approximately 1 hour. Following electrophoresis, the proteins were

transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, St Giles, UK)

using a wet system. The PVDF membrane was sequentially soaked in methanol, distilled

water and transfer buffer (20% (v/v) methanol, 192 mM Glycine, and 25 mM Tris HCl in

distilled water). Cassettes containing sponges, filter paper, the acrylamide gel and PVDF

membrane were assembled in the transfer apparatus (Bio-Rad) and they were immersed in

transfer buffer. The proteins were allowed to transfer to the PVDF membrane by applying

current (360 Amps) for 1 hour. Following transfer the membranes were incubated for

approximately 2 hours with 5% skimmed milk in Tris Buffered Saline with Tween (TBS-

T: 20 M Tris HCl pH 7.6, 0.14 M NaCl and 0.025% (v/v) Tween 80 in distilled water) in

order to block non specific sites. The membranes were incubated overnight at 4°C with

the primary antibody diluted in 5% skimmed milk in TBS-T. The information for the

primary antibodies used can be found in **Table 2-2**. The following day the membranes

were washed with TBS-T and were incubated for 1 hour at room temperature with

secondary antibody conjugated to horseradish peroxidase (HRP) (Dako, Glostrup,

Denmark) diluted 1 in 2,000 in 5% skimmed milk in TBS-T. Unbound antibody was

removed by washing with TBS-T and antigen-antibody complexes were visualised using

ECL+ chemiluminescence detection system (GE Healthcare). The membranes were put in

a cassette with X-ray film (Genetic Research Instrumentation, Essex, UK) in the dark

room and the film was developed using an X-ray film processor (Xograph, Imaging

Systems, Gloucestershire, UK). The expression of β-actin was quantified to assess protein

loading. The membranes were washed in TBS-T and were incubated for 1 hour with

mouse antibody against human β-actin (Sigma-Aldrich) diluted 1 in 10,000. The

membranes were washed with TBS-T and were incubated for 1 hour with rabbit anti-

mouse secondary antibody conjugated to horseradish peroxidase (Dako) diluted 1 in

2,000. The membranes were washed with TBS-T and the antigen-antibody complexes were visualised using SuperSignal West Substrate (Thermo Fisher Scientific, Cramlington, UK) on X-ray film. The expression of the protein of interest and of β-actin was quantified using the ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA) and the expression of the protein of interest was expressed as a ratio to the expression of β-actin. Protein extraction and detection by Western immunoblotting were carried out by Dr Laurence Loubière.

2.2.9   Immunohistochemistry

The experiments that are described in this section were carried out by Barbara Innes in Dr Judith Bulmer's laboratory in Newcastle, UK. Formalin-fixed paraffin-embedded sections (3 μm) of placental samples and placental bed biopsies were immunostained for MCT10, OATP1A2 and OATP4A1 using an avidin-biotin peroxidase technique (Vectastain Elite; Vector Laboratories, Peterborough, UK) (Barber *et al.*, 2005, Chan *et al.*, 2006). All reagents were prepared as per the kit instructions. Briefly, after dewaxing and rehydration the sections were processed in 0.1 M sodium citrate buffer (pH 6.0) in a pressure cooker for 60 seconds for antigen retrieval following by incubation with 1.6% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing in 0.1 M Tris/0.05 M saline (pH 7.6; TBS), the slides were overlaid with the supplied blocking serum, before incubation for 60 minutes at room temperature with primary antibody (details for antibody dilutions can be found in **Table 2-2**). Negative controls were performed for each sample by replacing the primary antibody with non-immune serum. After washing in TBS, the sections were incubated with biotinylated secondary antibody, washed with TBS and incubated with avidin-biotin-peroxidase complex. Following washes in TBS, the reaction was developed by incubation in 3,3'-

diaminobenzidine. Sections were lightly counterstained with Mayers haematoxylin, dehydrated, cleared and mounted in synthetic resin.

Table 2-2: *List of antibodies used for protein detection by Western immunoblotting (WB) and by immunohistochemistry (IHC).*

Protein	Antibody source	Species of immunised animal	Dilution for WB	Dilution for IHC
MCT8	Designed and validated in our lab (Ab4790)	Rabbit	1:500	Not applicable
MCT10	Gift from Prof F Verrey (Ramadan *et al.*, 2006)	Rabbit	1:500	1:250
LAT1	Gift from Dr P Taylor (Ritchie and Taylor, 2001)	Rabbit	1:500	Not applicable
CD98	Santa Cruz Biotechnology (C20: sc7095)	Goat	1:250	Not applicable
OATP1A2	Gift from Prof B Stieger (Huber *et al.*, 2007)	Rabbit	1 :500	1 :150
OATP4A1	Gift from Prof B Stieger (Huber *et al.*, 2007)	Rabbit	1:500	1:300

2.2.10 Statistical analysis

Data were analysed using the Minitab® statistical software (version 15). Analysis of variance (ANOVA) was performed using the general linear model followed by Tukey all pairwise multiple comparison post-hoc tests to assess differences between individual groups and taking into account all variables present as appropriate, i.e. gestation and placental type (IUGR or normal). For the mRNA expression data presented in this chapter, the residuals (differences between the observed values and the predicted values by the general linear model) did not pass the normality test as determined using the Kolmogorov-Smirnov test and, thus, required log transformation prior to statistical analysis.

2.3   Results

2.3.1   Validation of primer and probe sets

At first, the newly designed primers and probes were validated to ensure that the expression of the target genes could be detected accurately in duplex reactions with the control gene 18S.

Increasing concentrations of cDNA were amplified in multiplex reactions and the slope of the linear regression was calculated (**Figure 2-1**). The difference between the gradient of the regression line for 18S amplification and the gradient of the regression line for the amplification of each target cDNA was 0.07 for MCT10, 0.01 for LAT1 and 0.01 for CD98. Therefore, the amplification of these molecules could be accurately detected in multiplex with 18S. The values obtained for LAT2, OATP1A2 and OATP4A1 were 0.44, 0.29 and 0.71, respectively, indicating that 18S amplification may be interfering with the detection of these cDNAs. Therefore, the expression of these genes was assessed in singleplex reactions.
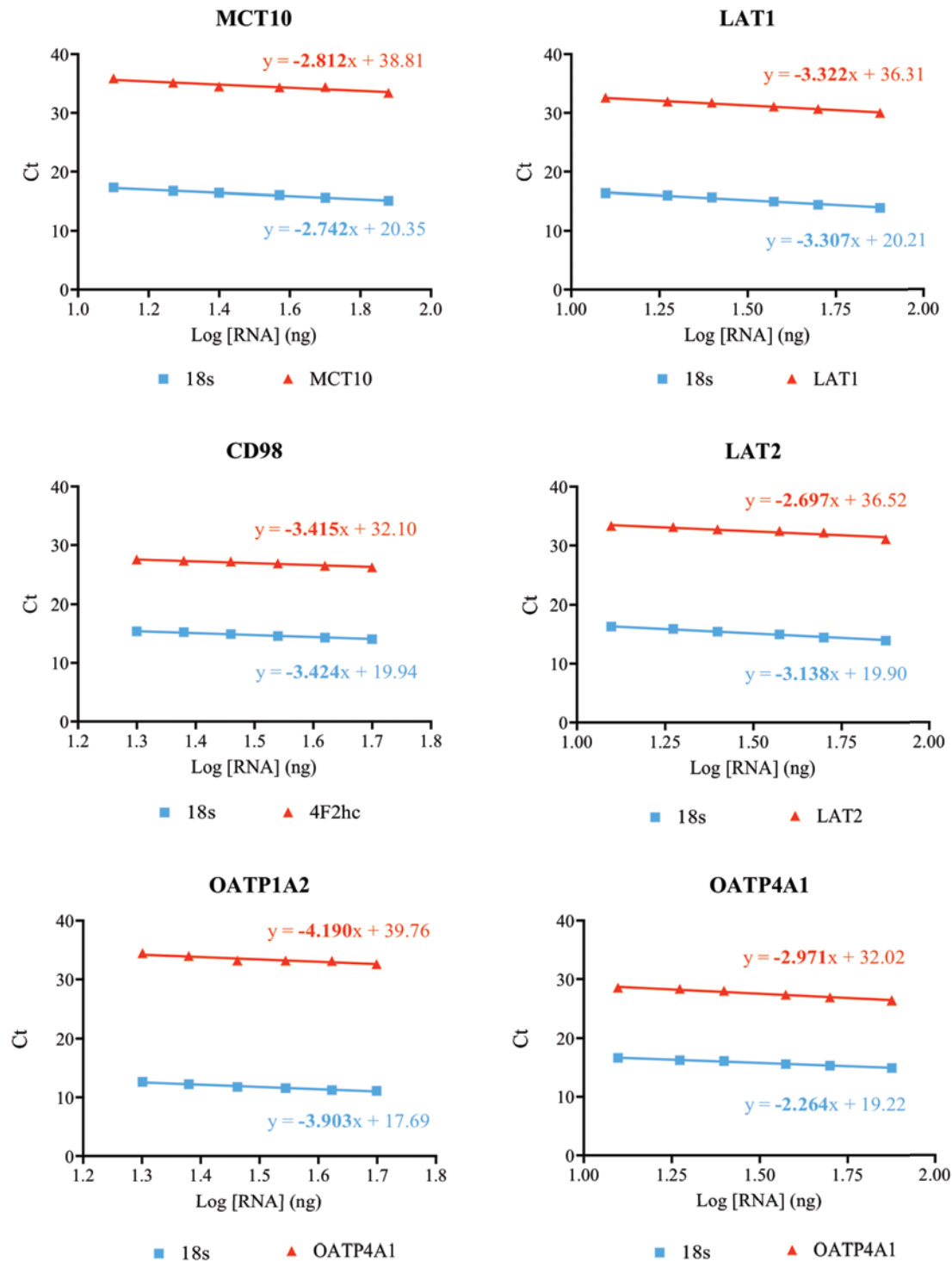
Figure 2-1: *Validation of the efficiency of primers and probes for MCT10, CD98, LAT1, LAT2, OATP1A2 and OATP4A1 in duplex reactions with the control 18S. Ct values are plotted against the concentration of RNA used. RNA was extracted from term placenta tissue. The equation of the linear regression line is also shown. Results from one representative experiment are shown. The results were confirmed in experiments with two different samples.*

2.3.2   Validation of the MCT8 antibody

The specificity of the MCT8 antibody was tested using term placenta homogenates and lysates of the MCT8-null choriocarcinoma cell line JEG-3 (Friesema *et al.*, 2006) transfected with the empty vector (VO) or with MCT8 as controls. In accordance with a previous study (Friesema *et al.*, 2006), MCT8 migrated at approximately 60 kDa. This band disappeared following pre-incubation of the antibody with the blocking (immunising) peptide (**Figure 2-2**).
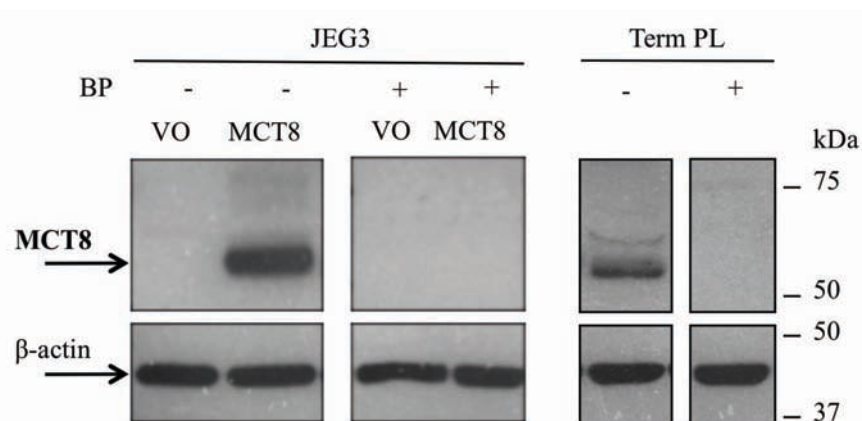
Figure 2-2: *Validation of the MCT8 antibody. The specificity of the MCT8 4790 polyclonal antibody was tested using MCT8-null JEG-3 cells transfected with either vector only (VO) or MCT8 and homogenates of term placenta tissue (Term PL). The samples were incubated with the MCT8 antibody pre-incubated with or without the blocking peptide (BP). Immunoreactivity for β-actin was used to assess protein loading.*

2.3.3   MCT8 and MCT10 expression in the human placenta across gestation

We assessed the relative mRNA expression of MCT8 and MCT10 in the human placenta from week 6 of gestation until term. The expression of both MCT8 and MCT10 increased with gestation (P<0.001 for both) (**Figure 2-3**). The expression of MCT8 at 6-10 weeks of gestation was 4-fold lower compared with term (P<0.001). At 11-14 weeks, MCT8 expression was 14-fold less than at term (P<0.001) and 10-fold less than at 27-34

weeks (P<0.01). At 15-20 weeks MCT8 expression was 22-fold less than at term (P<0.01). MCT10 expression at 6-10 weeks was 12-fold lower compared with term and 16-fold lower compared with 27-34 weeks (P<0.001 for both). At 11-14 weeks of gestation, MCT10 expression was 8-fold lower compared with term (P<0.001) and 13-fold lower compared with 27-34 weeks (P<0.01). At 15-20 weeks MCT10 expression was 6-fold lower compared with term (P<0.05).
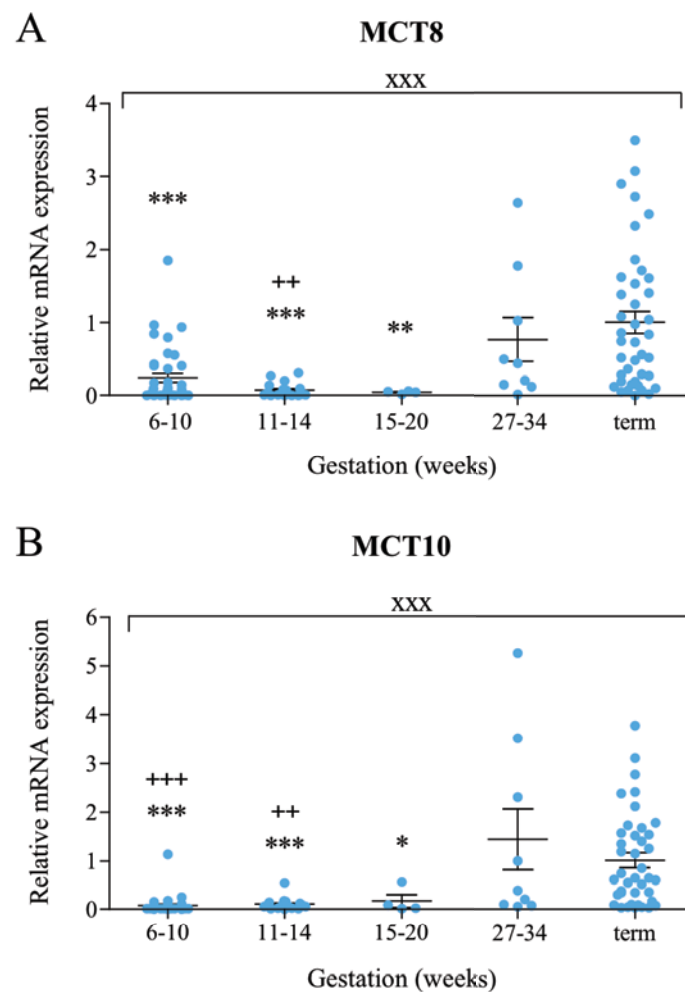
Figure 2-3: *Relative expression of mRNA encoding MCT8 **(A)** and MCT10 **(B)** in the human placenta from 6 weeks of gestation until term. Gene expression is compared with the mean expression at term that was given an arbitrary value of 1. Overall ANOVA results are indicated by xxx P<0.001. Differences in expression between individual groups were assessed by Tukey post-hoc tests. Statistically significant differences in gene expression compared to term are indicated by * (P<0.05), ** (P<0.01) and *** (P<0.001). Statistically significant differences compared to the 27-34 weeks group are indicated by ++ (P<0.01) and +++ (P<0.001). Lines represent means ±SEM. The data were log-transformed prior to analysis.*

The expression of MCT8 and MCT10 protein in the human placenta from week 8 of gestation until term was assessed by Western immunoblotting (**Figure 2-4** and **Figure 2-4**). Bands representing MCT8 protein were detected at all gestational ages as a single band at approximately 60 kDa (**Figure 2-4A**). Relative quantification of the bands revealed that MCT8 protein expression increased with gestation (**Figure 2-4B**; $P<0.001$). Expression in the first trimester was 68% less than at term ($P<0.001$) and 23% less than in the second trimester ($P<0.05$). Expression in the second trimester was 22% less than at term ($P<0.05$). MCT10 protein was detected from week 10 of gestation until term as a single band at approximately 50 kDa (**Figure 2-5A**) and was increased with gestational age, as demonstrated by relative densitometry (**Figure 2-5B**; $P<0.05$). In the first trimester MCT10 expression was 47% less than at term ($P<0.01$).
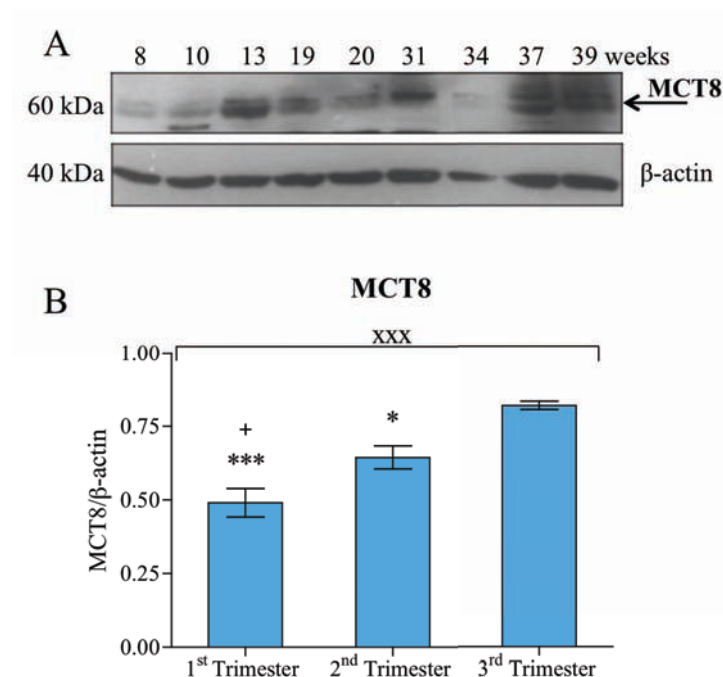
Figure 2-4: *Western immunoblotting **(A)** and relative densitometry **(B)** for MCT8 on placental homogenates from week 8 of pregnancy until term. Bands representing MCT8 were detected at approximately 60 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. The protein expression of MCT8 was quantified by relative densitometry and shown as a ratio to β-actin protein expression. Bars represent means ±SEM. Overall ANOVA results are indicated by xxx P<0.001. Statistically significant differences in protein quantification between groups assessed by Tukey post-hoc tests are indicated by *** (P<0.001) and * (P<0.05) when compared with term and by + (P<0.05) when compared with the 2nd trimester group.*
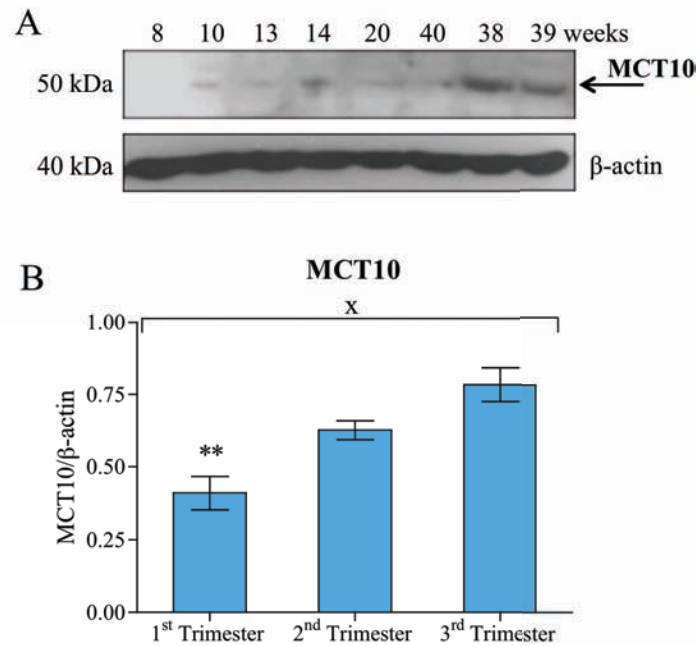
Figure 2-5: *Western immunoblotting **(A)** and relative densitometry **(B)** for MCT10 on placental homogenates from week 8 of pregnancy until term. Bands representing MCT10 were detected at approximately 50 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. The protein expression of MCT10 was quantified by relative densitometry and shown as a ratio to β -actin protein expression. Bars represent means ±SEM. Overall ANOVA results are indicated by x (P<0.05). Statistically significant difference in protein quantification between the 1ˢᵗ trimester and the term groups assessed by Tukey post-hoc tests is indicated by ** (P<0.01).*

We have also localised MCT10 protein in the human placenta using paraffin embedded sections from first trimester and third trimester villous placental biopsies and first trimester placental bed biopsies (**Figure 2-6**). Immunohistochemical analysis of first trimester biopsies indicated that MCT10 protein is present in both STs and CTs with more marked staining in the latter. Analysis of first trimester placental bed sections showed that MCT10 protein is also present in EVTs and decidual stroma. At term, MCT10 is mainly present in the ST cell layer. Throughout gestation there was also weak immunoreactivity within stromal cells of the villous placenta.
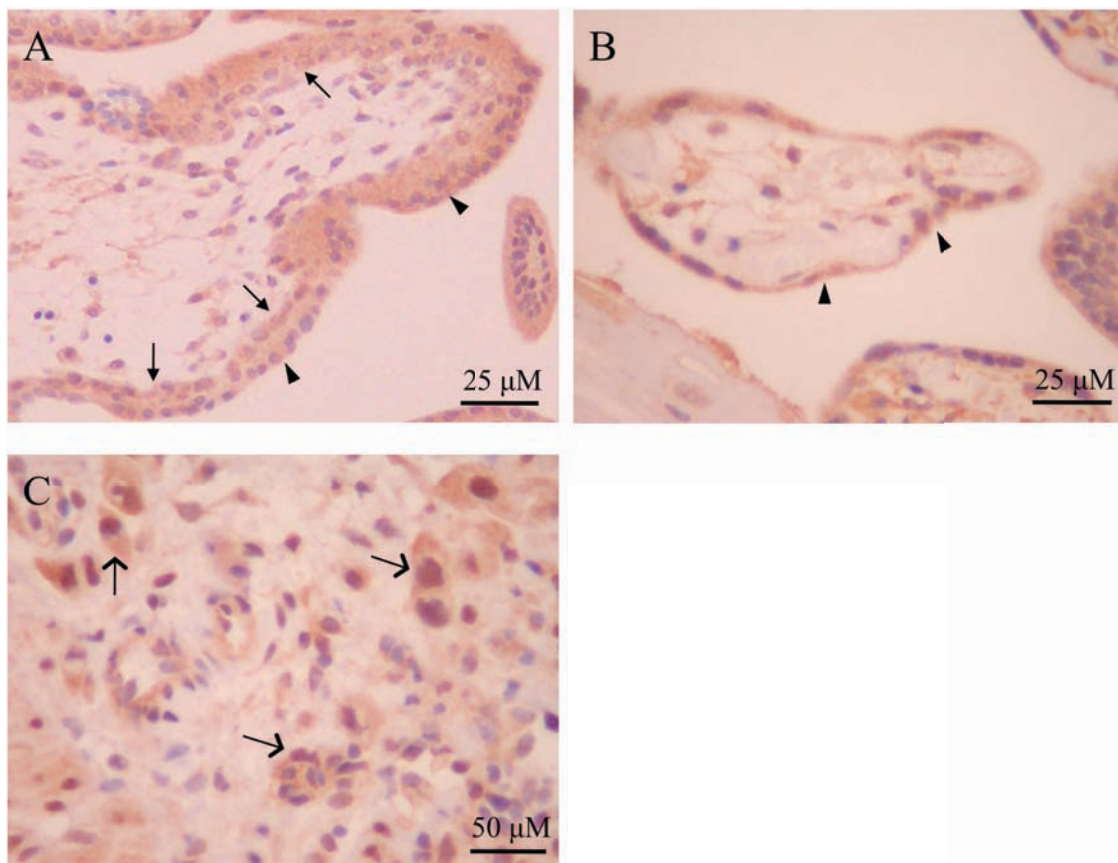
Figure 2-6: *Localisation of MCT10 protein in the human placenta. Immunoreactivity for MCT10 in representative sections obtained from first trimester (A) and term (B) villi and from first trimester placental bed (C). Positive CTs are indicated by closed arrows, STs by arrowheads and EVTs by open arrows.*

2.3.4   LAT1, LAT2 and CD98 expression in the human placenta across gestation

Overall, the mRNA expression of LAT1 and CD98 changed significantly with gestation ($P<0.001$ and $P<0.05$ respectively) (**Figure 2-7A and C**). The mRNA expression of LAT2 did not change with gestation (**Figure 2-7B**). The expression of LAT1 at 6-10 weeks, 11-14 weeks and 15-20 weeks was significantly lower than at term (2.5-fold; $P<0.001$, 3-fold; $P<0.001$ and 5-fold; $P<0.01$ respectively). Furthermore, the expression of LAT1 at 6-10 weeks and 11-14 weeks was significantly lower that at 27-34 weeks (2.8-fold; $P<0.05$ and 3.6-fold; $P<0.02$ respectively). The expression of CD98 was 3-fold lower at 11-14 weeks compared with term ($P<0.05$), but there was no difference at

6-10 weeks compared with term, suggesting that CD98 mRNA expression reaches a nadir at 11-14 weeks.
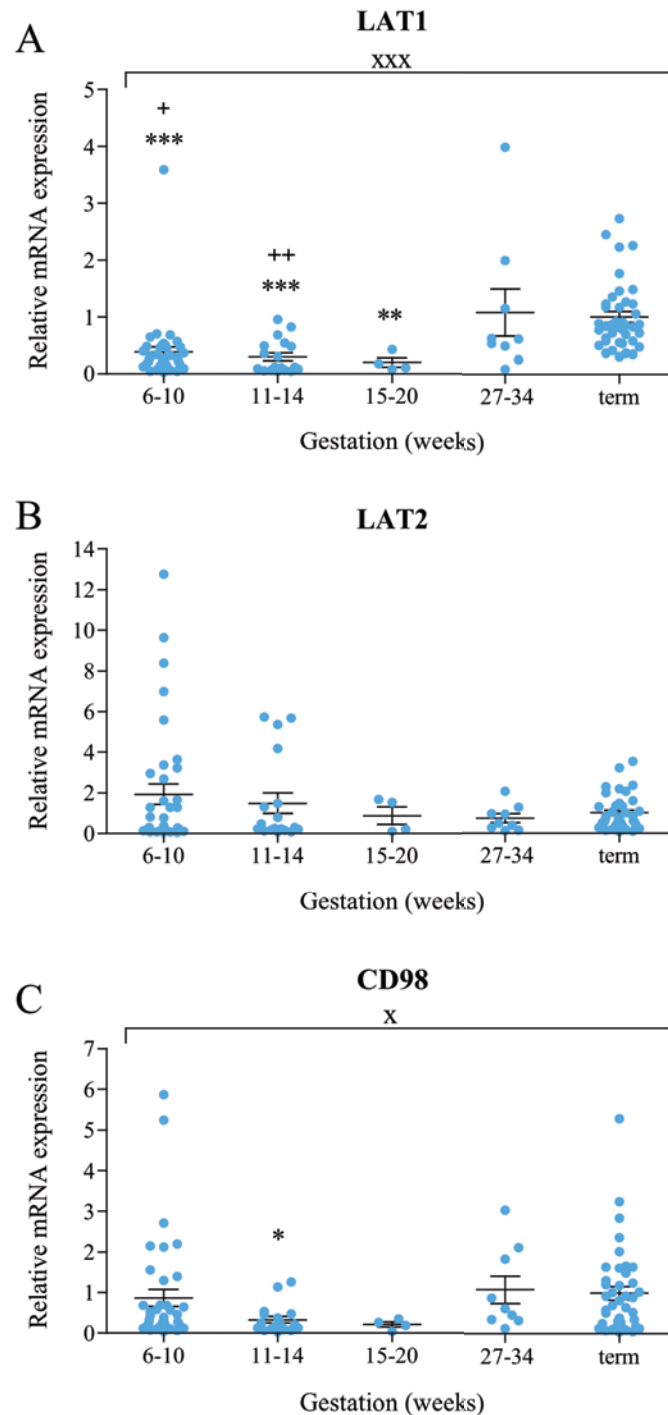
Figure 2-7: *Relative expression of mRNA encoding LAT1 **(A)**, LAT2 **(B)** and CD98 **(C)** in the human placenta from 6 weeks of gestation until term. Gene expression is compared with the mean expression at term that was given an arbitrary value of 1. Overall ANOVA results are indicated by xxx P<0.001 or x P<0.05. Differences in expression between individual groups were assessed by Tukey post-hoc tests. Statistically significant differences in gene expression compared to term are indicated by * (P<0.05), ** (P<0.01) and *** (P<0.001). Statistically significant differences compared to the 27-34 weeks group are indicated by + (P<0.05) and ++ (P<0.01). Lines represent means ±SEM. The data were log-transformed prior to analysis.*

The expression of LAT1 and CD98 proteins was determined by Western immunoblotting (**Figure 2-8**). Western immunoblotting experiments for LAT1 revealed the presence of a band representing the LAT1 monomer at approximately 30 kDa from week 32 of gestation. Western immunoblotting for CD98 revealed two bands at approximately 70 kDa and 100 kDa, probably corresponding to non-glycosylated and glycosylated forms of CD98 (Ritchie and Taylor, 2001). There was no significant difference in CD98 protein expression with gestational age (**Figure 2-8B2**).
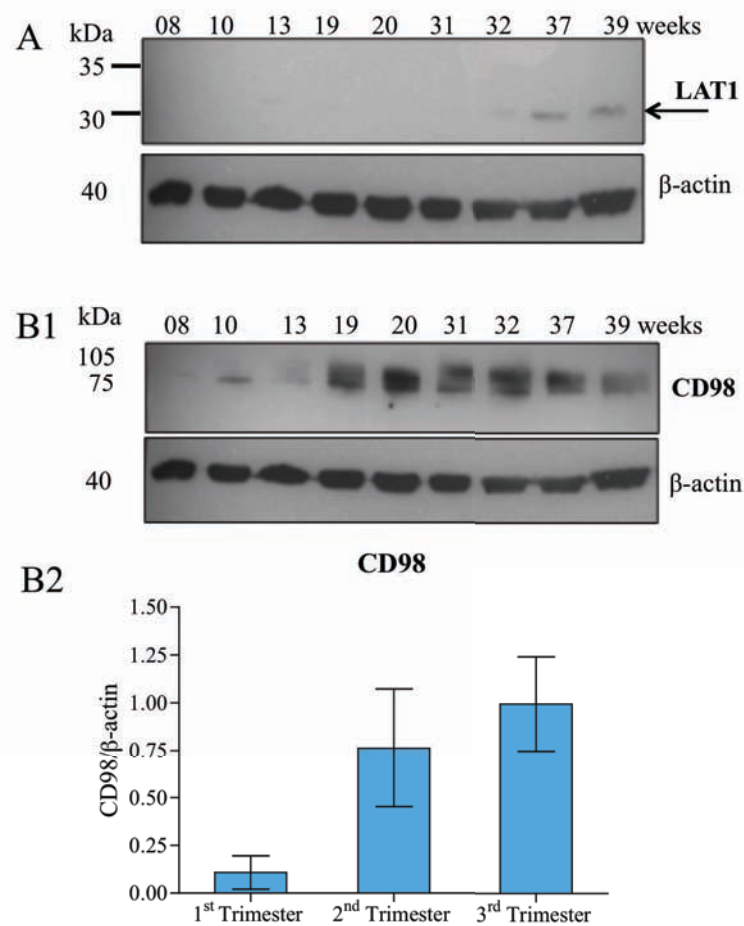
Figure 2-8: *Western immunoblotting for LAT1 **(A)** and CD98 **(B1)** on placental homogenates from week 8 of pregnancy until term. Bands representing LAT1 were detected at approximately 30 kDa. Bands for CD98 were detected at approximately 70 and 100 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. **(B2)** CD98 protein expression was quantified by relative densitometry and shown as a ratio to β-actin protein expression. Bars represent means ±SEM.*

2.3.5   OATP1A2 and OATP4A1 expression in the human placenta across gestation

Overall, the mRNA expression of OATP1A2 and OATP4A1 changed significantly across gestation ($P<0.05$ and $P<0.01$, respectively) (**Figure 2-9**). The expression of OATP1A2 at 6-10 weeks was significantly lower than at term (1.8-fold; $P<0.05$). The expression of OATP4A1 was significantly lower than at term only at 11-14 weeks (5-fold; $P<0.05$) but not at 6-10 weeks, indicating that OATP4A1 expression is lowest at 11-14 weeks.
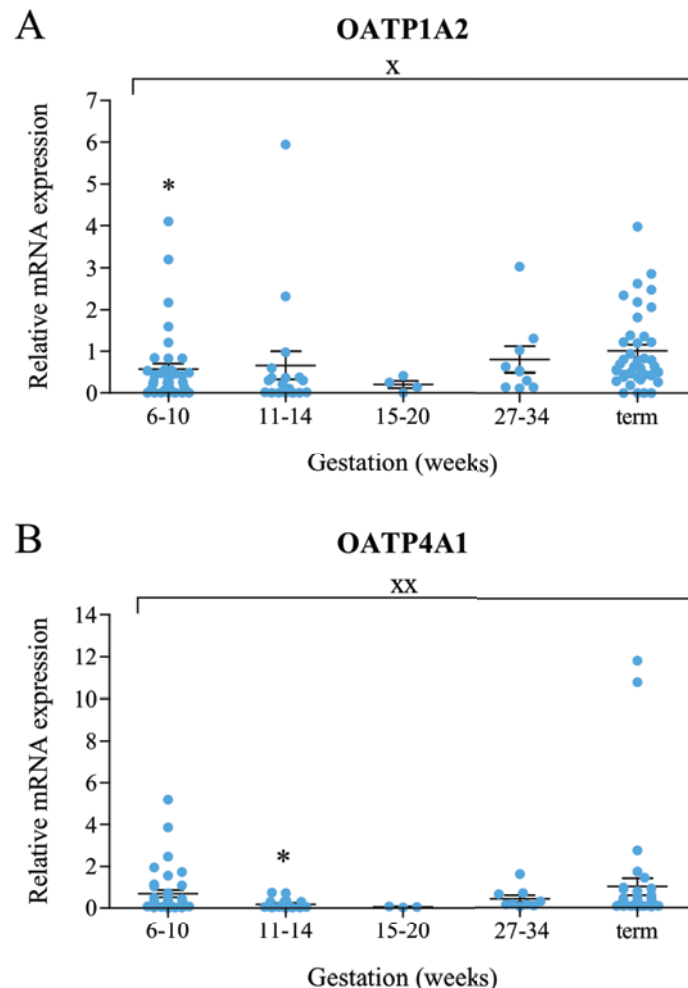
Figure 2-9: *Relative expression of mRNA encoding OATP1A2 **(A)** and OATP4A1 **(B)** in the human placenta from 6 weeks of gestation until term. Gene expression is compared with the mean expression at term that was given an arbitrary value of 1. Overall ANOVA results are indicated by x (P<0.05) and xx P<0.01. Differences in expression between individual groups were assessed by Tukey post-hoc tests and statistically significant differences in gene expression compared to term are indicated by * (P<0.05). Lines represent means ±SEM. The data were log-transformed prior to analysis.*

The presence of OATP1A2 and OATP4A1 protein in the human placenta was confirmed by Western immunoblotting. Both proteins were detected from week 8 of gestation until term (**Figure 2-10** and **Figure 2-11**). Western immunoblotting for OATP1A2 revealed multiple bands between 60 and 85 kDa representing different glycosylated forms of the protein (Gao *et al.*, 2000, Kullak-Ublick *et al.*, 1997). OATP4A1 was detected as a single band at approximately 60 kDa (Kullak-Ublick *et al.*, 1995). There was no significant change in the protein expression of OATP1A2 or OATP4A1 with gestational age.
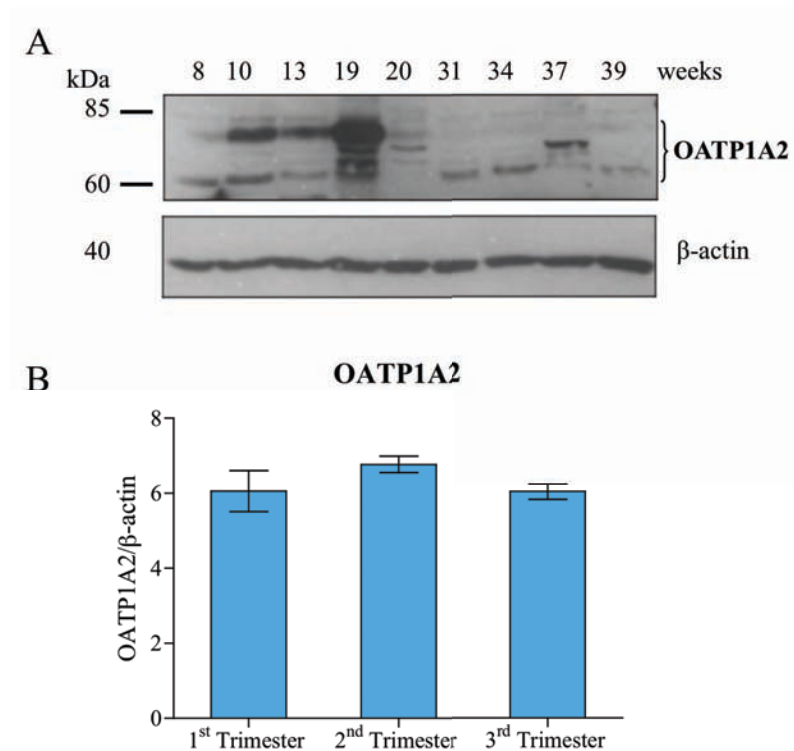
Figure 2-10: *Western immunoblotting **(A)** and relative densitometry **(B)** for OATP1A2 on placental homogenates from week 8 of pregnancy until term. Bands representing different glycosylation states of OATP1A2 were detected between 60 and 85 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. OATP1A2 protein expression was quantified by relative densitometry and shown as a ratio to β -actin protein expression. Bars represent means ±SEM.*
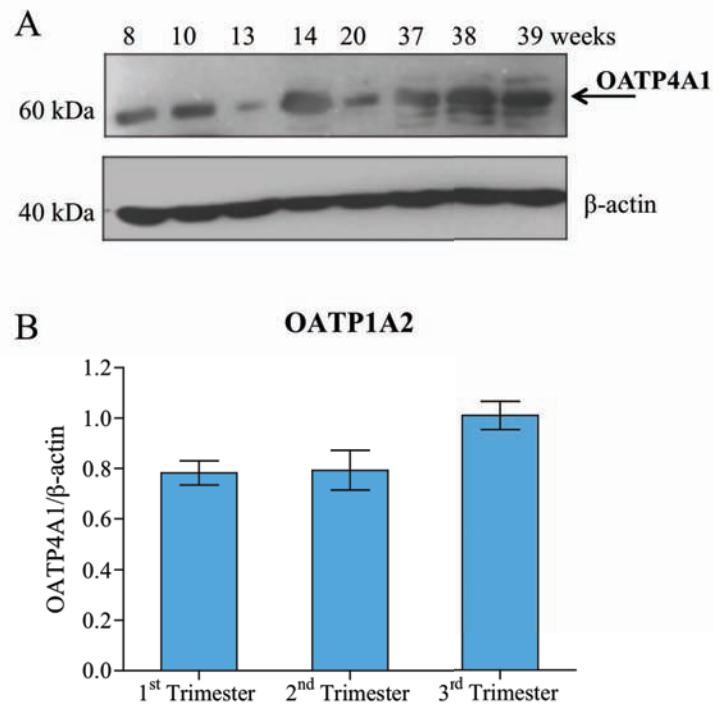
Figure 2-11: *Western immunoblotting (A) and relative densitometry (B) for OATP4A1 on placental homogenates from week 8 of pregnancy until term. OATP4A1 was detected as a single band at approximately 60 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. OATP4A1 protein expression was quantified by relative densitometry and shown as a ratio to β-actin protein expression. Bars represent means ±SEM.*

OATP1A2 and OATP4A1 protein localisation was examined by immunohistochemistry (**Figure 2-12**). OATP1A2 protein was strongly detected in both villous CTs and STs in placental sections from first trimester and term biopsies. OATP1A2 protein was also present EVTs. In contrast to OATP1A2, immunohistochemistry for OATP4A1 protein revealed strong staining in STs but weak staining in CTs and EVTs.
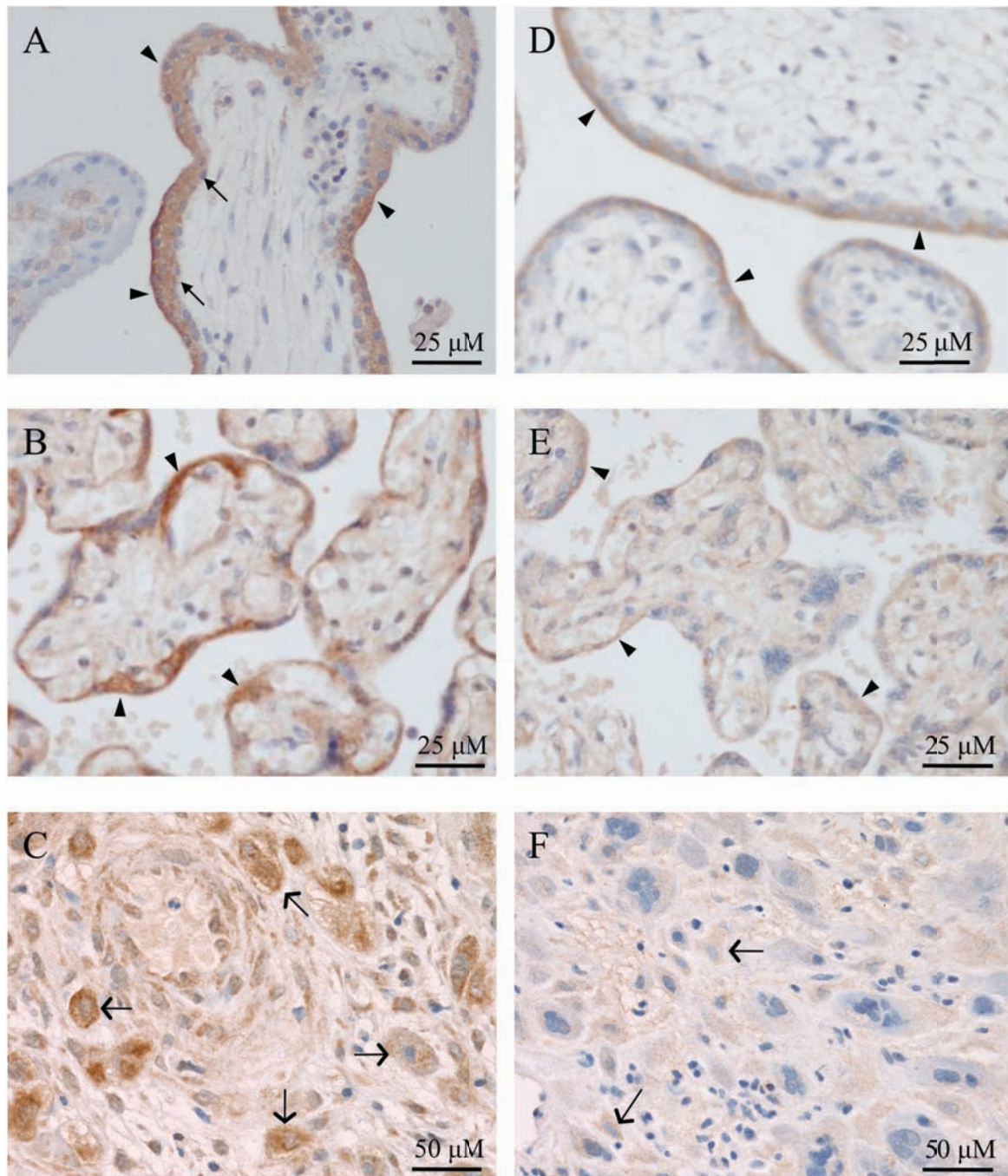
Figure 2-12: *Localisation of OATP1A2 (**A**, **B** and **C**) and OATP4A1 (**D**, **E** and **F**) protein in the human placenta. Immunoreactivity for OATP1A2 and OATP4A1 in representative sections obtained from first trimester (**A** and **D**) and term (**B** and **E**) villi and from first trimester placental bed (**C** and **F**). Positive CTs are indicated by closed arrows, STs by arrowheads and EVTs by open arrows.*

2.3.6   Changes in the expression of TH transporters with IUGR

Previous data suggest that the mRNA and protein expression of MCT8 in the human placenta is increased in IUGR pregnancies (Chan *et al.*, 2006). We therefore assessed whether there were any changes in the placental expression of TH transporters in normal pregnancies and in pregnancies complicated by IUGR. We compared the expression of TH transporters in biopsies from placentae obtained immediately after delivery in the early third trimester (25-34 weeks) or at term (37-41 weeks).

In contrast to the results obtained previously (Chan *et al.*, 2006), in this expanded cohort there was no difference in the mRNA expression of MCT8 in normal compared with IUGR placentae. However, the expression of MCT8 protein was increased in early onset IUGR samples compared with gestationally matched normal controls (P<0.001; **Figure 2-13**). There was no difference in MCT8 protein expression between late onset IUGR samples and normal samples delivered at term. According to the overall ANOVA results, the mRNA expression of MCT10 was reduced with IUGR (P<0.01; **Figure 2-14A**). However there were no statistically differences when the expression between individual groups was compared by Tukey post-hoc tests. Assessment of MCT10 protein expression by Western immunoblotting did not reveal any significant difference in the protein expression of MCT10 between normal and IUGR placental samples (**Figure 2-14B**). The mRNA expression of OATP4A1 was different between normal and IUGR samples as determined by the overall ANOVA analysis (**Figure 2-16B**; P<0.05). However, no statistically significant differences were found when the mRNA expression of OATP4A1 between individual groups was compared by Tukey post-hoc tests. The mRNA expression of LAT1, LAT2, CD98 and OATP1A2 did not change significantly with IUGR (**Figure 2-15** and **Figure 2-16**).

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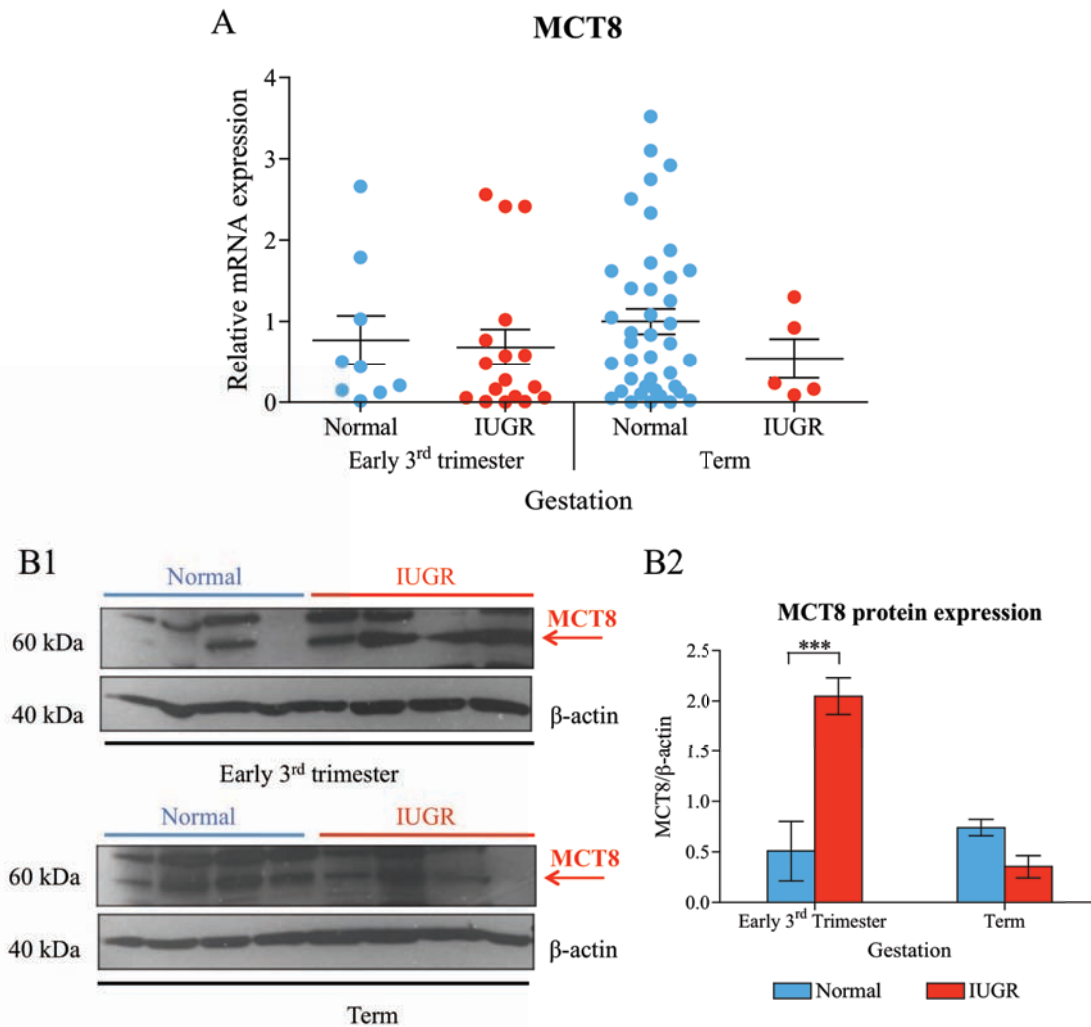
Figure 2-13: *Changes in MCT8 expression with IUGR. (A) Relative expression of mRNA encoding MCT8 was assessed in placental samples from normal pregnancies and from pregnancies complicated by IUGR that were delivered in the early third trimester or at term. Gene expression is compared with the mean expression in normal term samples that was given an arbitrary vlaue of 1. Lines are representative of the mean ±SEM for each group. The data were log-transformed prior to analysis. (B1) Representative Western immunoblotting for MCT8 on randomly selected placental homogenates from normal and IUGR pregnancies delivered in the early third trimester or at term. A band representing MCT8 was detected at approximately 60 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading. (B2) MCT8 protein expression was quantified by relative densitometry and shown as a ratio to β-actin protein expression. Bars represent means of four normal and four IUGR samples per gestation ±SEM. Statistically significant differences are indicated by *** (P<0.001).*
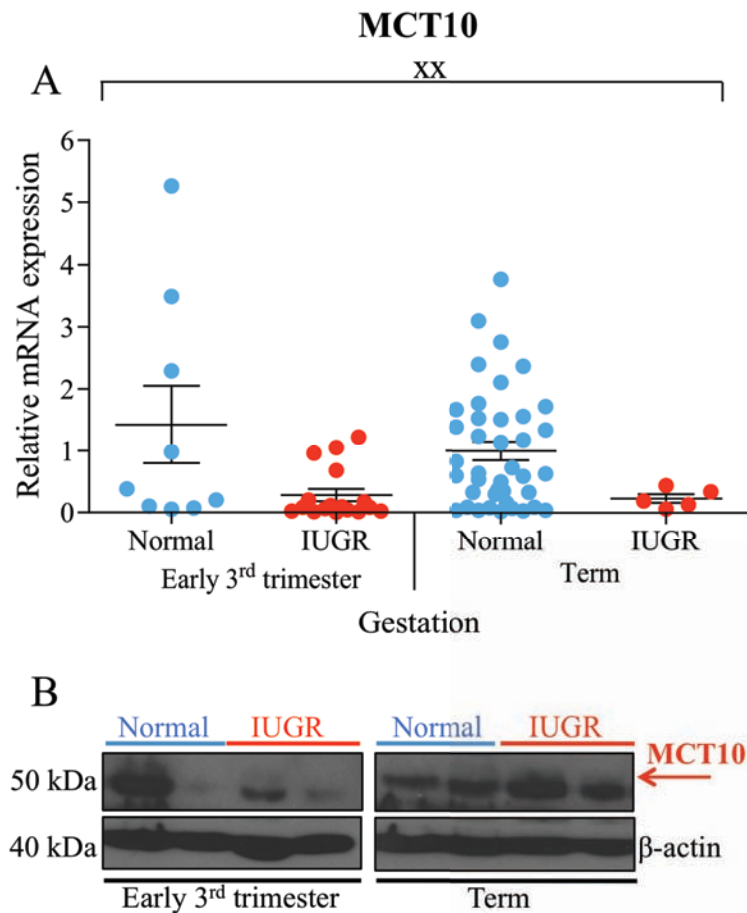
Figure 2-14: *Changes in MCT10 expression with IUGR. **(A)** Relative expression of mRNA encoding MCT10 was assessed in placental samples from normal pregnancies and from pregnancies complicated by IUGR that were delivered in the early third trimester or at term. Gene expression is compared with the mean expression in normal term samples that was given an arbitrary value of 1. Lines represent means ±SEM. Overall ANOVA results between normal and IUGR samples are indicated by xx P<0.01. The data were log-transformed prior to analysis. **(B)** Western immunoblotting for MCT10 on randomly selected placental homogenates from normal and IUGR pregnancies delivered in the early third trimester or at term. A band representing MCT10 was detected at approximately 50 kDa. Immunoblotting for β-actin on the same membrane was used to assess protein loading.*
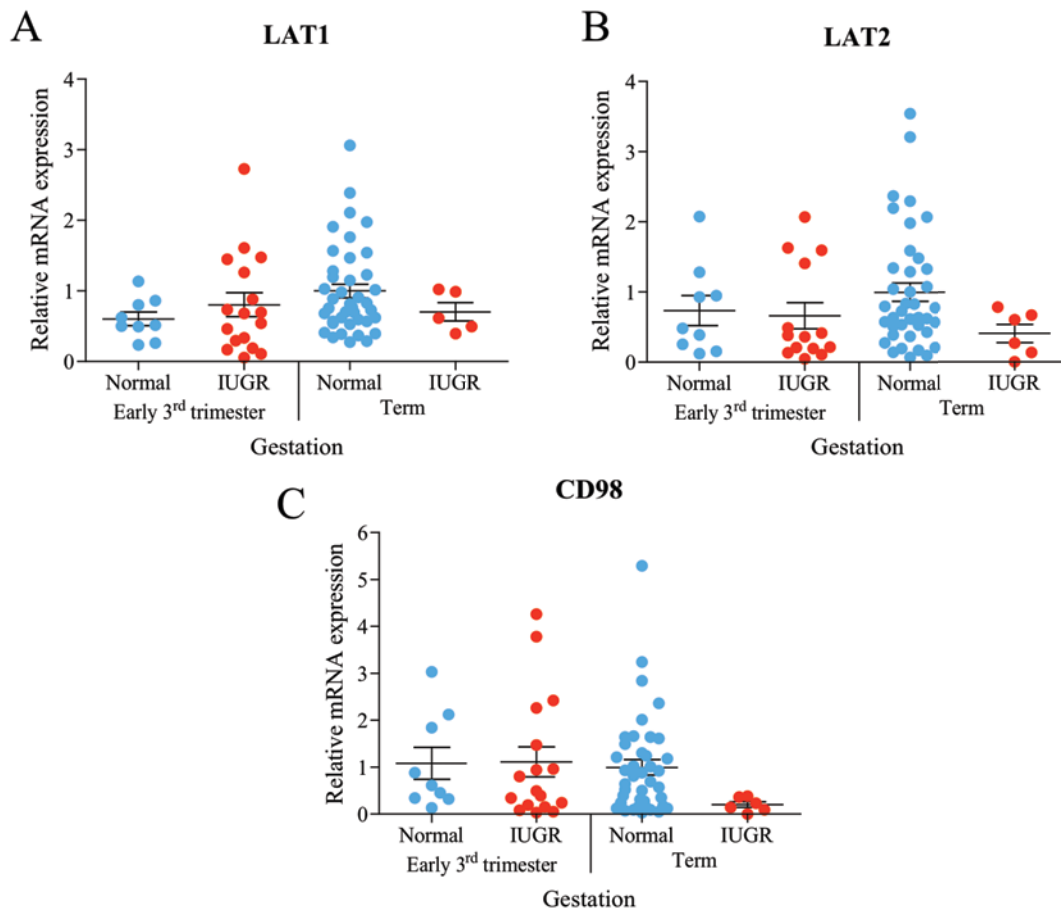
Figure 2-15: *Changes in the mRNA expression of LAT1 (A), LAT2 (B) and CD98 (C) with IUGR. Relative expression of mRNA encoding LAT1, LAT2 and CD98 was assessed in placental samples from normal pregnancies and from pregnancies complicated by IUGR that were delivered in the early third trimester or at term. Gene expression is compared with the mean expression in normal term samples that was given an arbitrary value of 1. Lines represent means ±SEM. The data were log-transformed prior to analysis.*
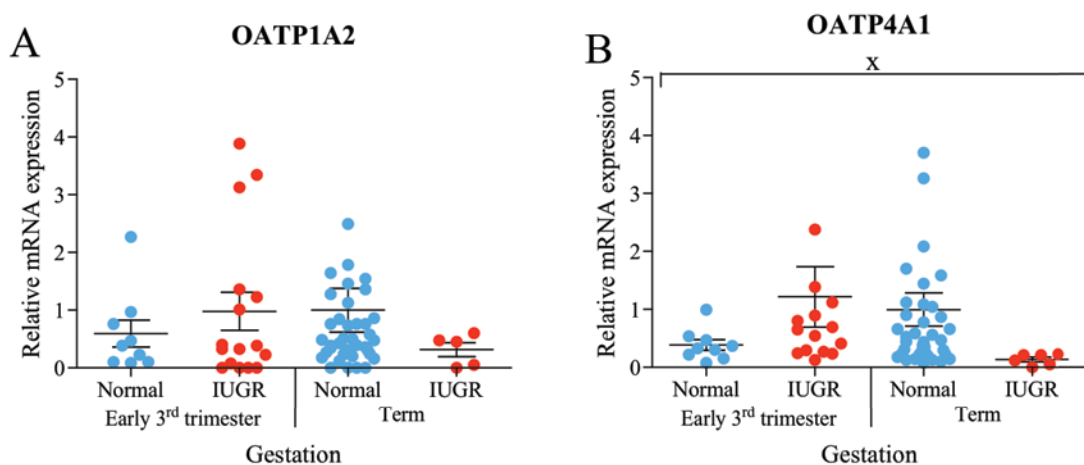
Figure 2-16: *Changes in the mRNA expression of OATP1A2 (A) and OATP4A1 (B) with IUGR. Relative expression of mRNA encoding OATP1A2 and OATP4A1 was assessed in placental samples from normal pregnancies and from pregnancies complicated by IUGR that were delivered in the early third trimester or at term. Gene expression is compared with the mean expression in normal term samples that was given an arbitrary value of 1. Overall ANOVA results between normal and IUGR samples are indicated by x P<0.05. Lines represent means ±SEM.. The data were log-transformed prior to analysis.*

2.4   Discussion and conclusions

2.4.1   The ontogeny of TH transporters in the human placenta

This study demonstrates that a range of TH transporters are expressed in the human placenta from the first trimester until term and they show different patterns of expression with increasing gestational age. MCT8, MCT10, LAT1 and OATP1A2 mRNA expression increases with advancing gestation, indicating that there might be increased fetal and placental demand for maternal THs and other compounds transported by these proteins as the pregnancy progresses. The mRNA expression of CD98 and OATP4A1 reaches a nadir between weeks 11 and 14 of gestation, which coincides with the onset of fetal TH production (Morreale de Escobar *et al.*, 2004), whilst the mRNA expression of LAT2 remains constant throughout gestation. Furthermore, the protein presence of these transporter proteins in the human placenta throughout gestation was demonstrated (with the exception of LAT2) and MCT10, OATP1A2 and OATP4A1 proteins were localised in specific cell types in the human placenta and placental bed.

Our group has previously demonstrated that MCT8 mRNA is expressed in the human placenta throughout gestation and its expression increases with gestational age (Chan *et al.*, 2006). We have also demonstrated the presence of MCT8 protein in the human placenta throughout gestation and we have localised MCT8 protein in villous CTs and STs throughout gestation and in decidual stromal cells and EVTs in first and second trimester placental bed biopsies. In light of evidence that MCT8 is one of the transporters responsible for both TH uptake and efflux (Friesema *et al.*, 2003, Friesema *et al.*, 2008), these results suggest that MCT8 can facilitate TH uptake and efflux by the majority of placental cell types. MCT8 may therefore mediate the effects of TH on trophoblast

differentiation and survival (Barber *et al.*, 2005, Canettieri *et al.*, 2008, Laoag-Fernandez *et al.*, 2004, Maruo *et al.*, 1991, Maruo *et al.*, 1995, Matsuo *et al.*, 1993) and contribute to transplacental transport of TH, which is essential for fetal development. In the present work, we have repeated the assessment of the expression levels of MCT8 in the human placenta at the mRNA and protein level due to significantly increased numbers in our placental sample cohort. Our results support our previous findings that MCT8 is expressed from week 6 of gestation until term and its expression increases as the pregnancy progresses.

MCT10 is known to transport aromatic amino acids and to be primarily responsible for their efflux as demonstrated in studies with *Xenopus* oocytes (Kim *et al.*, 2002, Ramadan *et al.*, 2006). It has recently been shown that like MCT8, MCT10 can also transport THs and facilitate both TH uptake and efflux (Friesema *et al.*, 2008). Although it has been shown before that MCT10 is present in the human placenta (Park *et al.*, 2005), this is the first study to describe the expression of MCT10 mRNA and protein throughout gestation and localise MCT10 expression in the human placenta. We have found that MCT10 mRNA and protein expression increases with gestation, suggesting an increased demand for TH and aromatic amino acids that are essential for fetoplacental growth. Localisation of MCT10 protein by immunohistochemistry showed that MCT10 is expressed in EVTs, villous CTs and villous STs and decidual stromal cells during the first trimester and is mainly expressed in the ST layer at term. These results suggest that, like MCT8, MCT10 may facilitate uptake of THs from the maternal circulation necessary for placental development and for transport to the fetal circulation. However, TH transport by MCT10 is subject to competitive inhibition by aromatic amino acids (Friesema *et al.*,

2008), which are also necessary for fetoplacental growth and this has to be considered when investigating its contribution to TH uptake.

Several studies have demonstrated the expression of LAT1, LAT2 and their obligate heterodimer CD98 in the human placenta (Prasad *et al.*, 1999, Ritchie and Taylor, 2001, Rossier *et al.*, 1999, Yanagida *et al.*, 2001). System L is an important mechanism for amino acid exchange and in addition transports THs (Friesema *et al.*, 2001, Ritchie *et al.*, 2003, Ritchie and Taylor, 2001). According to our results, placental LAT1 mRNA expression increases with gestation, whilst the mRNA expression of LAT2 does not change across pregnancy. The mRNA expression of CD98 reaches a nadir at weeks 11-14 of gestation and it reaches the highest level at term. Recruitment of more samples is necessary to assess more accurately the expression of CD98 at 15-20 weeks. In the limited samples available, CD98 mRNA expression was the lowest at 15-20 weeks of gestation, however this observation did not reach statistical significance possibly due to low number of samples available for this gestation. This is in accordance with a previous study that described an increase in the protein expression of LAT1 and CD98 at term compared with mid-trimester (12-21 weeks) (Okamoto *et al.*, 2002). LAT1 and LAT2 are functional only as heterodimers with the heavy chain glycoprotein CD98 (Pineda *et al.*, 1999, Prasad *et al.*, 1999). Therefore, the transport of amino acids and THs by the system L can be regulated by changes in the expression of either of the components of the heterodimers. We have demonstrated the presence of CD98 protein in the human placenta from week 8 of gestation until term. However, we were only able to detect LAT1 protein after week 32 of gestation. Since LAT1 has been previously detected in mid-trimester human placenta (Okamoto *et al.*, 2002), it is possible that failure to detect LAT1 protein in second trimester samples is due to a low affinity of the antibody used. Due to the lack

of a reliable antibody for the detection of LAT2 protein at the time of this study, we were unable to show protein expression of LAT2. In previous studies, LAT1 and CD98 have been localised in the ST layer of the human placenta (Okamoto *et al.*, 2002, Ritchie and Taylor, 2001). Such findings are consistent with a role for LAT1 in transplacental transport of amino acids and THs.

Assessment of the expression of OATP1A2 and OATP4A1 in the human placenta revealed an increase in the mRNA expression of OATP1A2 with gestation whilst the expression of OATP4A1 reached a nadir between weeks 11 and 14 of gestation. It has to be noted that for both OATP1A2 and OATP4A1, mRNA expression was lower at 15-20 weeks, however this result was not statistically significant possibly due to the low number of samples available for this gestation. It would therefore be useful to recruit more samples from this gestation in order to assess more accurately the expression of TH transporters at 15 to 20 weeks of gestation. In addition, we have shown that OATP1A2 and OATP4A1 proteins are present in the human placenta throughout gestation. Our findings are consistent with previous studies that have demonstrated mRNA and protein expression of OATP1A2 and OATP4A1 in the human placenta (Briz *et al.*, 2003, Patel *et al.*, 2003, Sato *et al.*, 2003). In the study by Patel *et al* the authors investigated the mRNA expression of OATP1A2 and OATP4A1 in a relatively small sample cohort from first trimester (n=8) and term (n=6) placentae and they have shown that whilst OATP4A1 expression is the same in the first trimester and at term, OATP1A2 expression is reduced at term compared with first trimester. In terms of OATP4A1 our results confirm these findings since what we observed was that OATP4A1 expression was reduced between weeks 11 and 14 of gestation but not between 6 and 10 weeks. In contrast, our results for the expression of OATP1A2 show an opposite trend. However, it has to be noted that our

data were obtained from a much larger cohort of placental biopsies. Localisation by immunohistochemistry revealed the presence of OATP1A2 protein in villous CTs, STs and EVTs, consistent with a role for this protein in uptake of THs and a variety of other organic compounds. OATP4A1 was predominantly localised in the ST layer both in the first trimester and at term, consistent with previous reports that have reported OATP4A1 expression in the ST of term placenta (Sato *et al.*, 2003). Furthermore, there was low expression of OATP4A1 in EVTs.

2.4.2   Changes in the expression of TH transporters with IUGR

An additional part of this study was to assess the expression levels of the TH transporters in human placentae from pregnancies complicated by early onset and late onset IUGR compared with gestationally matched controls. Our results demonstrate that although there was no difference in MCT8 mRNA expression between groups, there was increased protein expression of MCT8 in placentae from early onset IUGR compared with gestationally matched controls. Furthermore, there were significant differences in the expression of MCT10 and OATP4A1 between IUGR and normal samples at the mRNA level, however there were no significant differences when gestationally-matched groups were compared. There was no difference in the mRNA expression of the other TH transporters with IUGR.

Our finding that MCT8 protein expression is increased with early onset IUGR is in accordance with our previously published observations (Chan *et al.*, 2006). However, in contrast to what we have previously reported, we found no differences in the mRNA expression of MCT8 with IUGR possibly due to increased number of samples that were included in this cohort. It is possible that the expression of MCT8 protein is regulated

post-transcriptionally, thus accounting for the discrepancy between the mRNA and protein data. This is the first report that investigates the expression of MCT10 with IUGR. Unpublished data by Cleal *et al* demonstrate that MCT10 mRNA expression in placentae from normal pregnancies positively correlates with birth weight, head circumference and lean mass (Cleal, 2009), thus supporting our observation that there is a trend of decreased MCT10 mRNA expression in the IUGR placenta. There are no previous reports of changes in the expression of OATP4A1 with IUGR. Therefore, the expression of MCT10 and OATP4A1 should be assessed in the future with more samples in order to conclusively determine whether the expression of these transporters is altered with IUGR. Our data show no differences in the mRNA expression of LAT1, LAT2 and CD98 with IUGR. Previous data have shown that in IUGR there is reduced transport of the amino acids leucine and lysine (Jansson *et al.*, 1998). It is possible that the expression of L-System amino acid transporters is altered post-transcriptionally and/or post-translationally in IUGR resulting in reduced amino acid and TH transport.

IUGR is most commonly associated with malplacentation (Khong, 1989). It has been shown that in IUGR the numbers of endovascular EVTs are reduced (Huppertz *et al.*, 2006) and the transformation of maternal spiral arteries is defective (Brosens *et al.*, 1977, Brosens *et al.*, 1972). Furthermore, there is increased apoptosis within the ST (Endo *et al.*, 2005, Ishihara *et al.*, 2002, Smith *et al.*, 1997) and increased syncytialisation of CTs (Newhouse *et al.*, 2007) in IUGR compared with normal placentae. T_3 promotes invasion and motility (Barber *et al.*, 2005, Canettieri *et al.*, 2008, Maruo *et al.*, 1991, Maruo *et al.*, 1995, Matsuo *et al.*, 1993, Oki *et al.*, 2004) and downregulates apoptosis (Laoag-Fernandez *et al.*, 2004) in EVT-like cell lines and EVTs isolated from first trimester placentae. Therefore, changes in TH transport early in pregnancy, which is a critical

period for normal placentation, may be contributing to the malplacentation associated with IUGR. Unfortunately, the clinical manifestation of IUGR can only be diagnosed later in pregnancy. It is thus impossible to assess the expression of the molecular apparatus regulating TH transport in first trimester placentae from IUGR pregnancies.

It has been shown by our group and others that the serum concentration of free T_3 and free T_4 is reduced in IUGR fetuses compared with gestationally matched controls (Kilby *et al.*, 1998, Thorpe-Beeston *et al.*, 1991). The mechanisms that are responsible for the reduced TH levels with IUGR have not been elucidated yet. At term, the THs that are found in the fetal circulation are a mixture of maternal THs supplied by transplacental transport and of THs produced by the developing fetus. Therefore, the reduced concentration of THs in the fetal circulation may be due to reduced fetal TH production and/or inefficient transplacental transport of maternal THs. It is also possible that the IUGR placenta has a reduced capacity for TH transport due to its decreased size. Changes in the expression of TH transporters and deiodinase enzymes could also affect transplacental transport of THs. Our group has shown before that there are no differences in placental deiodinase enzyme activity with IUGR, indicating that deiodinase enzymes are not responsible for the hypothyroxinaemia observed in IUGR fetuses (Chan *et al.*, 2003). Reduced activity of System L with IUGR (Jansson *et al.*, 1998) may contribute to the reduced level of circulating THs in the IUGR fetus. In this thesis, a trend of decreased MCT10 mRNA expression was observed with IUGR, which could also contribute to decreased transplacental transport of THs. The increased MCT8 expression may be an attempt of the IUGR placenta to compensate for the decreased TH transport, however, it may not be adequate to restore transplacental TH transport to normal levels.

2.4.3 Conclusions

The findings presented in this chapter demonstrate the presence of a range of TH transporters in the human placenta throughout gestation. These transporters comprise a system that is in place to ensure adequate supply of THs and other substrates essential for the development of the fetoplacental unit. Preferential expression of these transporters in different cell types in conjunction with deiodinase enzyme expression can regulate the uptake and efflux of THs, thus ensuring the availability of the optimal TH levels necessary for normal fetal and placental development. Our data suggest that there are changes in the placental expression of TH transporters in IUGR pregnancies suggesting that transplacental TH transport may be altered. This may be a contributing factor to the malplacentation associated with IUGR and to the reduced serum levels of THs in IUGR fetuses. Abnormal levels of THs in the fetal circulation could be partly responsible for the increased risk for fetal and neonatal mortality and morbidity with IUGR.

Chapter 3: T$_3$ Responsiveness and Transport by Primary Cytotrophoblasts from Normal and IUGR Human Term Placentae

3.1   Introduction

IUGR is a pregnancy complication that is characterised by failure to achieve the genetic growth potential of a fetus (Scifres and Nelson, 2009) and is a major contributor to fetal and neonatal morbidity and mortality (Kok *et al.*, 1998, Tan and Yeo, 2005). IUGR can arise due to multiple factors of maternal, placental, fetal or environmental origin (Sankaran and Kyle, 2009) and is commonly associated with uteroplacental insufficiency (Mayhew *et al.*, 2003, Teasdale, 1984). The remodelling of the maternal spiral arteries that facilitates unobstructed blood flow from the maternal circulation towards the placenta in normal pregnancies is defective in IUGR pregnancies (Brosens *et al.*, 1977, Brosens *et al.*, 1972). Furthermore, histological examination of third trimester placentae has revealed that the number of apoptotic nuclei within villous STs is increased in IUGR compared with normal placentae (Endo *et al.*, 2005, Ishihara *et al.*, 2002, Smith *et al.*, 1997). The differentiation of villous CTs to STs by a process termed syncytialisation, is also increased in IUGR as demonstrated in experiments using a placental explant model (Crocker *et al.*, 2004, Newhouse *et al.*, 2007) or primary cultures of term CTs (Newhouse *et al.*, 2007).

Maternal TH status is one of several factors that are thought to be involved in human placental development. Untreated maternal hyperthyroidism has been associated with complications of malplacentation, including IUGR, placental abruption and pre-eclampsia (Mestman, 2004), whilst maternal subclinical hypothyroidism has been associated with increased risks of miscarriage, placental abruption and preterm delivery (Abalovich *et al.*, 2002, Casey *et al.*, 2005). *In vitro*, T₃ has been shown to increase the invasive ability of first trimester EVTs and EVT-like cell lines (Barber *et al.*, 2005,

Maruo *et al.*, 1991, Oki *et al.*, 2004) and suppress their apoptosis (Laoag-Fernandez *et al.*, 2004).

Our group has previously reported that the serum concentration of free T_4 and free T_3 is reduced in IUGR fetuses (Kilby *et al.*, 1998). Furthermore, we have shown that there is increased protein expression of the TR isoforms TRα1, TRα2 and TRβ1 in the term placenta with IUGR (Kilby *et al.*, 1998). As described in the previous chapter, the protein expression of the TH transporter MCT8 is increased with early onset IUGR, whilst there was a trend of decreased mRNA expression of the TH transporter MCT10 in the placenta with IUGR. Previously published data by our group have demonstrated that there is no difference in the activity of the deiodinase enzymes D2 and D3 in the placenta with IUGR, suggesting that TH metabolism is not altered with IUGR (Chan *et al.*, 2003).

The finding that the expression of TRs in the placenta is altered with IUGR suggests that the IUGR placenta may have altered sensitivity to TH. Furthermore, the changes in the expression of the TH transporters MCT8 and MCT10 with IUGR suggest that there may be differences in trophoblast uptake of TH and in TH transplacental transport with IUGR. Primary cultures of CTs from term placenta are a useful model to study placental biology. After plating, these isolates form monolayers of mononucleate cells. With time in culture the cells syncytialise to form multinucleate CTs that are thought to be representative of STs (Greenwood *et al.*, 1996). Cultures of isolated CTs can thus be used to investigate CT behaviour as well as transport by STs *in vitro*. In this study, this cell model was used to assess the T_3 responsiveness and transport in CTs isolated from placentae from normal pregnancies (normal CTs) or from pregnancies complicated by IUGR (IUGR CTs).

3.2 Materials and Methods

3.2.1 Sample collection

This study had the approval of the Local Research Ethics Committee (South Birmingham) and the Research and Development Committee of the Birmingham Women's Hospital.

Placentae from normal (n=27) and late onset IUGR (n=14) pregnancies were collected following delivery by caesarean section. All were delivered after 35 completed weeks of gestation, as determined by a first trimester ultrasound scan of crown-rump length. The IUGR cases were diagnosed prospectively using ultrasound and they had at least two of the following four characteristics: (i) abdominal circumference, measured by ultrasound, less than the tenth centile for gestation, (ii) abdominal circumference growth velocity of less than 1.5 standard deviation over 14 days, (iii) oligohydramnios defined as amniotic fluid index $\leq$ tenth centile for gestation and (iv) absent or reversed end diastolic flow velocity in the umbilical artery Doppler studies. The IUGR fetuses were not known to have abnormal karyotypes. None of the pregnancies was complicated by maternal hypertension.

3.2.2 Isolation and culture of CTs from term placenta tissue

Immediately after delivery by caesarean section, the placentae were transferred to the laboratory at room temperature, where they were processed to isolate CTs following the method previously published by Kliman (Kliman *et al.*, 1986) and modified by Greenwood (Greenwood *et al.*, 1996).

The placenta was processed in the tissue culture cabinet using autoclaved instruments and glassware and sterile solutions to avoid any contamination. All the solutions were warmed to 37°C prior to usage. The edges of the placenta, the umbilical cord region and any calcified areas were discarded. The placenta was cut into approximately one-inch cubes and placed in a glass beaker with approximately 300 ml of 0.9% saline. The cubes were retrieved one at a time and the chorionic plate and the decidual cell layer were removed and discarded. The villous material was dissected into small pieces. Any vessels found in the tissue were removed. The villous material was washed in a beaker with fresh saline and was passed through a sterile gauge. The tissue was crudely minced using scissors and any unwanted material, such as vessels, decidua pieces and blood clots, was removed. The tissue was washed two more times in fresh saline and then weighed.

The tissue (30-32 g) was added to 150 ml Hepes Buffered Salt Solution (HBSS: 5.40 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.34 mM Na_2HPO_4 heptahydrate, 5.55 mM glucose and 25 mM HEPES; pH 7.4) containing 30 mg of DNAse and 2,250 units of trypsin without EDTA, calcium or magnesium (Invitrogen, Paisley, UK) in a 1 L conical flask. The flask was placed in a shaking water bath at 37°C and was shaken at 80 oscillations per minute for 30 minutes. After 30 minutes, the flask was placed horizontally in the hood and the tissue was allowed to settle at the bottom of the flask. The supernatant (100 ml) was transferred to a glass beaker. Fresh HBSS (100 ml containing 20 mg of DNAse and 1,500 units of trypsin) was added to the flask and the flask was returned to the shaking water bath for another 30 minutes. The supernatant was carefully layered on top of 5 ml Newborn Calf Serum in four 50 ml tubes and the tubes were centrifuged for 10 minutes at 2,200 × g with brake off. The resulting pellets consisted of CTs and other

digested cells. They were resuspended in 1 ml Dulbecco's modified Eagles medium with 25 mM glucose (DMEM) and they were pooled together. Once the second incubation was complete, another 100 ml of supernatant were carefully removed and 75 ml of fresh HBSS containing 15 mg DNAse and 1,125 units of trypsin were added. The tissue was incubated in the shaking water bath for another 30 minutes. The freshly harvested supernatant was centrifuged as before. At the end of the final incubation as much of the supernatant as possible was removed without taking any tissue and was centrifuged as before. All the pellets were pooled together and DMEM was added to a total volume of 50 ml. The solution was centrifuged at $2,200 \times g$ for 10 minutes with brake off.

The resulting pellet was resuspended in 6 ml DMEM with high glucose and was carefully layered on top of percoll gradients in order to separate the CTs according to cellular density. The percoll gradients were made from previously prepared stock solutions ranging from 70% to 10% percoll in HBSS. They were layered in 30 ml glass tubes as shown in **Figure 3-1** The tubes were centrifuged at 2,800 rpm for 30 minutes with brake off. Following centrifugation the top bands were discarded and the bands between 35% and 55% percoll, that contained the CTs, were collected in a 50 ml tube. The volume was made up to 50 ml with CT culture medium, which was prepared using equal amounts of DMEM (with 25 mM HEPES and 25 mM glucose; Invitrogen) and F12 nutrient mixture (with 10 mM glucose; Invitrogen) and was supplemented with 10% (v/v) Fetal Calf Serum (FCS; Invitrogen), 1,000 units/L Penicillin (Invitrogen), 0.001% (w/v) Streptomycin (Invitrogen), 0.005% (w/v) Gentamicin and 0.029% (w/v) L-Glutamine (Invitrogen). The cell suspension was centrifuged at 2,200 rpm for 10 minutes. The pellet was resuspended in 2 ml CT culture medium and the number of cells was counted under a microscope using a haemocytometer. Usually the yield was between 60 and 100×10^6

cells. The final concentration of glucose in the CT culture medium was 17.5 mM. Although this concentration of glucose is hyperglycaemic, according to a study by Hahn *et al.* cell viability and secretion of hCG by cytotrophoblasts are unaffected by culture in media with low (5.5 mM) or high (25 mM) glucose (Hahn *et al.*, 1998).
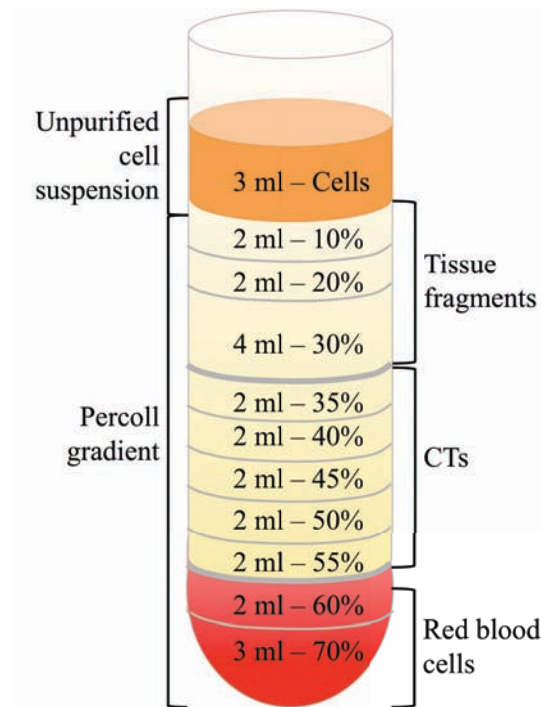
Figure 3-1: Percoll gradient. A gradient ranging from 70% to 10% was layered in order to separate the cells on the basis of size. The cells were layered on top of the percoll gradient and centrifuged. The CTs were found in the five bands between 55 and 35% and they were visualised as pale white cell layers.

The cells were resuspended in CT culture medium to a final concentration of 1.5×10^6 cells per ml and they were transferred to tissue culture plates at a density of 3×10^6 cells per well of a 6-well plate. They were incubated in a CO_2 incubator at 37°C under normoxic conditions with 5% CO_2. The following day the cultures were washed three times with phosphate buffered saline (PBS) containing magnesium chloride and calcium chloride to remove dead and floating cells. Fresh CT culture medium was added and the cells were returned to the incubator. The cells were washed twice with PBS daily and

were maintained for up to four days after isolation. The protocol for CT isolation is summarised in **Figure 3-2**.
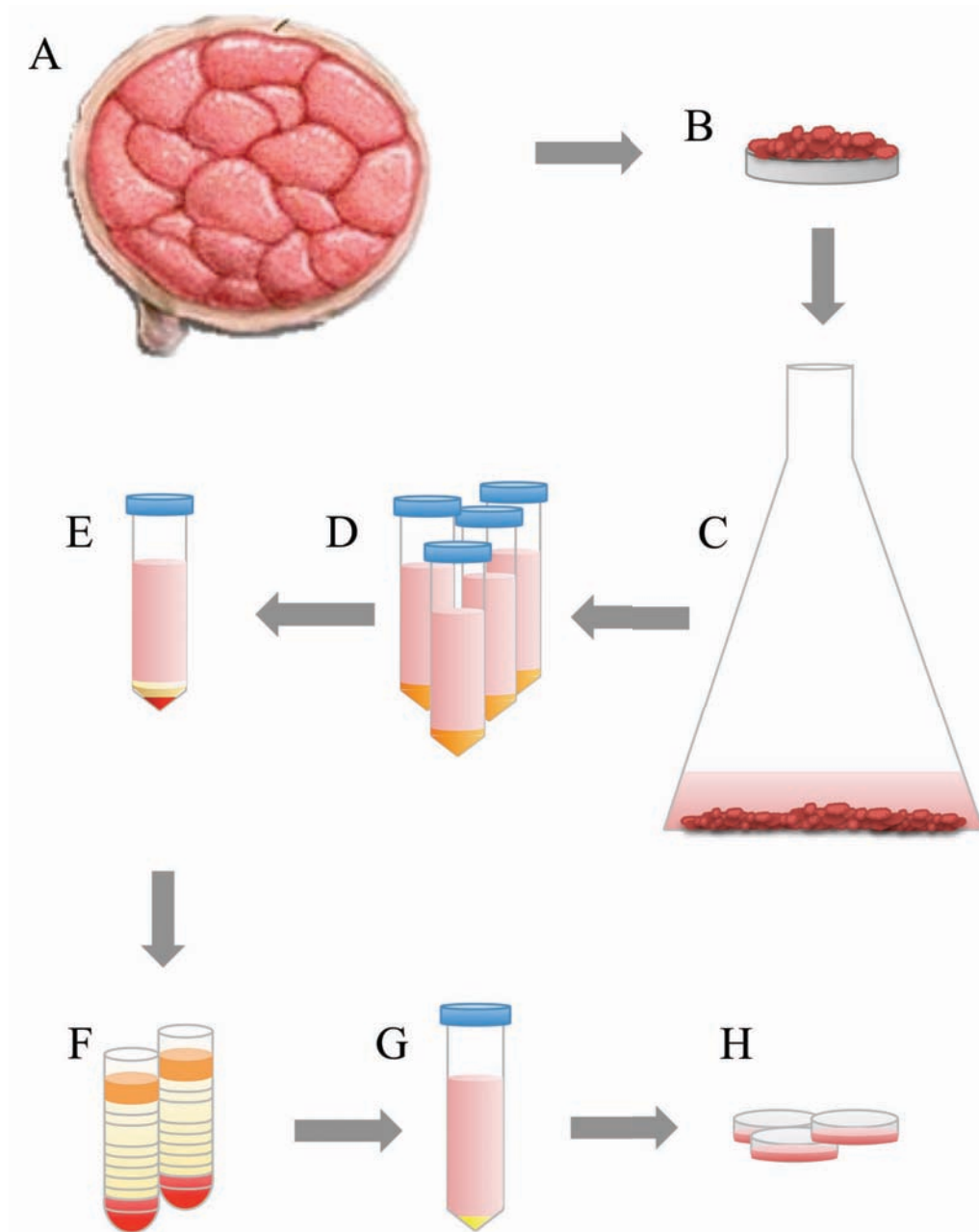
Figure 3-2: *Isolation of CTs. **(A)** A placenta was collected immediately after delivery by caesarian section. **(B)** The villous part of the tissue was disected into small pieces. **(C)** The tissue was taken through three rounds of trypsinisation. **(D)** The supernatant was collected after each trypsinisation and centrifuged on top of newborn calf serum. **(E)** The pellets were collected in a tube with DMEM and centrifuged. **(F)** The pellet was resuspended in 6 ml DMEM and was overlayed on top of two percoll gradients and centrifuged for separation of the different cell types. **(G)** The cells in the layers between 55 and 35% were collected, resuspended in CT culture media and centrifuged. **(H)** The final pellet was resuspended in CT culture media and following cell counting the cells were plated in tissue culture dishes.*

3.2.3   Assessment of cell purity by immunofluorescence

In order to assess cell purity the cells were stained with antibodies against cytokeratin-7 and vimentin. Cytokeratin-7 is a marker for cells of epithelial origin, including trophoblast cells, while vimentin is a marker for mesenchymal cells and stromal decidua (Maldonado-Estrada *et al.*, 2004). Therefore, CT cells should be positive for cytokeratin-7 and negative for vimentin.

CTs were plated in 24-well plates at a density of 0.75×10^6 cells per well. Three days after isolation the cells were washed in PBS and they were fixed and permeabilised by incubating for 25 minutes in 100% methanol at -20°C. The methanol was then removed and the cells were washed three times with PBS and stored in PBS at 4°C until they were stained. The cells were incubated for 30 minutes in blocking solution (10% v/v calf serum in PBS) in order to block sites for non-specific binding. They were incubated with mouse antibodies against human cytokeratin-7 (Sigma-Aldrich; clone LDS-68; 1:50) or vimentin (Sigma-Aldrich; clone V9; 1:100) for 1 hour at room temperature and washed three times with PBS. Secondary goat anti-mouse IgG antibody labelled with green-fluorescent Alexa Fluor 488 dye (1:250; Invitrogen) and blue-fluorescent Hoechst 33258 stain (Sigma-Aldrich; 1:1,000) were added for 1 hour. The cells were washed three times with PBS and stored in PBS at 4°C until they were examined using an inverted fluorescent microscope. Pictures from three fields per well were captured and the percentage of cytokeratin-7 positive and vimentin positive cells was calculated as a measure of cell purity. Isolated CTs usually consist of a relatively pure population. The purity of CT cultures was checked regularly and the cultures were consistently $\geq$ 95% pure.

3.2.4   Assessment of cell syncytialisation

3.2.4.1   Immunofluorescent staining for desmosomes

The fusion of mononucleate CTs that results in the formation of syncytialised multinucleate cells is complete by 66 to 90 hours after plating the cells. Cell syncytialisation can be visualised using antibodies against cell junction proteins, such as desmosomes. By counting the total number of nuclei and the number of nuclei that are found in a multinucleate cell (a cell with three or more nuclei), one can estimate the percentage of nuclei that are found in syncytia. For this purpose the cells were stained with mouse antibody against human desmosomal protein (Sigma-Aldrich; clone ZK-31; 1:200) followed by secondary goat anti-mouse IgG antibody labelled with green-fluorescent Alexa Fluor 488 dye (1:250; Invitrogen) and blue-fluorescent Hoechst 33258 stain (Sigma-Aldrich; 1:1,000) using the procedure described in section 3.2.3.

3.2.4.2   hCG secretion

In vitro, syncytialisation correlates with increased secretion of hCG by CTs (Kliman *et al.*, 1986). Secretion of hCG was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (DRG, Marburg, Germany). The cell medium was collected daily before washing the cells in PBS and it was stored at -20°C. Protein was also harvested from the same wells with 2% (w/v) SDS. The samples were thawed and mixed thoroughly and 25μl of each sample and of standard solutions of hCG (5, 50, 200, 500 and 1,000 mIU/ml) were added in duplicate to separate microtiter wells coated with anti-β-hCG antibody. Anti-hCG antiserum conjugated to horseradish peroxidase was added to the wells (100μl). The samples were thoroughly mixed for 10 seconds and incubated at room temperature for 30 minutes. Then, the contents were

shaken out of the wells and the wells were rinsed five times with distilled water. The samples were incubated with 100 μl of substrate solution containing tetramethylbenzidine. After 10 minutes the reaction was stopped using the provided solution of 0.5 M sulphuric acid and the optical density of the samples at 450 nm was assessed using a Victor[3] multilabel counter (Perkin Elmer). The standard curve was used to determine the hCG concentration of the samples. The concentration of the protein harvested from the same wells was estimated using the Bio-Rad Dc protein assay as described in section 2.2.7. Secretion of hCG was then expressed as mlU of hCG, per hour in culture, per ml of medium and was normalised per mg of protein in order to account for differences in cell density with time.

3.2.5 T_3 treatment

In order to assess the impact of T_3 on CTs from normal and IUGR pregnancies, the cells were treated with increasing doses of T_3 for 48 and/or 72 hours. Following isolation the cells were plated in CT culture medium supplemented with 10% charcoal-stripped fetal calf serum (SFCS; First Link, Birmingham, UK). Charcoal-stripped serum is filtered through charcoal to remove non-polar material, such as hormones, including THs that are normally present in FCS. After an overnight incubation during which the cells were allowed to adhere, the cells were washed three times with PBS and fresh CT culture medium supplemented with 10% SFCS and T_3 (0, 1, 10 and 100nM) was added. The cells were washed daily with PBS and incubated in fresh T_3 supplemented medium.

3.2.5.1 *Cell survival*

Cell survival in response to T_3 was assessed using the MTT assay. Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced to purple

formazan in living cells. Therefore, this assay can provide a relative measure of the number of viable cells. As shown by Barber *et al*, CTs isolated from term placenta do not proliferate with time in culture (Barber *et al.*, 2005). Therefore, any changes in cell number would reflect differences in cell survival.

CTs were plated in a 96-well plate at a density of 3×10^5 cells per well and they were treated with T_3 for 48 hours as described above. The assay was performed 48 hours after T_3 treatment. MTT was added to the cells at a final concentration of 0.5 mg per ml. The cells were incubated with MTT at 37°C for 3 hours. The medium was removed and the formazan was solubilised by the addition of 100 μl dimethyl sulphoxide. The absorbance of the coloured solution was quantified by measuring the absorbance of each sample at 570 nm using a plate reader (Bio-Tek). The background absorbance at 690 nm was also measured and subtracted from the absorbance at 570 nm. Four replicates were performed within each experiment. The effect of T_3 was assessed on CTs from nine normal and five IUGR pregnancies. The results were normalised to the average absorbance for 0 nM T_3 treatment within each experiment.

3.2.5.2   Apoptosis

3.2.5.2.1   Caspase 3/7 activity assays

The effect of T_3 on apoptosis was assessed using an assay that measures the activity of the effector caspases 3 and 7 (Caspase-Glo 3/7 Assay; Promega). The luminogenic substrate provided is cleaved by active caspase 3 or 7 resulting in the production of a substrate for luciferase and thus in luciferase activation and light production.

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The cells were plated in a white-walled 96-well plate at a density of 3×10^5 cells per well and they were treated with T_3 for 48 hours as described in section 3.2.5. The reagent provided with the kit and the cells were allowed to equilibrate to room temperature before performing the assay. The reagent was added to the cells (100 μl) and to wells containing only CT media (blank control). The contents of the wells were mixed using a plate shaker at 350 rpm for 30 seconds. The plate was incubated at room temperature for 2 hours. The luminescence of each sample was determined using a plate reader (Victor³; Perkin Elmer). Each treatment was performed in triplicate and the effect of T_3 was assessed using CTs from nine normal and five IUGR placentae. The results were normalised to the average luminescence for 0 nM T_3 treatment within each experiment.

3.2.5.2.2 *Immunofluorescent staining for M30*

Apoptosis was also assessed by immunofluorescent staining for M30. M30 is an epitope that is revealed when cytokeratin-18 is cleaved by caspase-3 (Kadyrov *et al.*, 2001). Using an antibody targeting M30 the number of nuclei with perinuclear M30 staining can be counted in order to determine the number of apoptotic cells.

CTs were plated in a 24-well plate at a density of 7.5×10^5 cells per well and they were treated with T_3 for 48 hours as described in section 1.1.1.5. The cells were fixed for assessment by immunofluorescence and stained as described in section 3.2.3. The cells were stained with an antibody against M30 (1:50; Roche Diagnostics, West Sussex, UK) followed by staining with secondary anti-mouse IgG antibody labelled with green-fluorescent Alexa Fluor 488 dye (1:250; Invitrogen) and with blue-fluorescent Hoechst 33258 stain (Sigma-Aldrich; 1:1,000). Using an inverted fluorescent microscope pictures

were captured from three fields per well using a 20× objective. The number of nuclei with M30 perinuclear staining was expressed as a percentage of the total number of nuclei within each field. The results were normalised to the average percentage for 0 nM T_3 treatment within each experiment. Each treatment was performed in duplicate. M30 staining was assessed in CTs from four normal and three IUGR placentae by one investigator blinded to placenta type and treatment.

The above techniques for assessment of cell survival and apoptosis were validated for use in this cell model prior to proceeding with the described experiments. The cells were treated with increasing doses (0-1,000 μM) of H_2O_2 that is known to induce apoptosis in CTs (Moll *et al.*, 2007). Cell survival and apoptosis were assessed 48 hours following H_2O_2 treatment, using the MTT assay, the caspase 3/7 activity assay and immunofluorescent staining for M30 as described above.

3.2.5.3 hCG secretion

The effect of T_3 on hCG secretion was assessed as described in section 3.2.4.2. The cells were plated in a 12-well plate at a density of 1.5×10^6 cells per well and they were treated with T_3 as described in section 3.2.5. They were washed daily with PBS. Cell media and protein were harvested at 18, 66 and 90 hours post-culture, corresponding to 0, 48 and 72 hours after T_3 treatment. The secretion of hCG was assessed and the results were expressed as the percentage change in hCG secretion between 18 and 66 hours and between 18 and 90 hours post-culture. The secretion of hCG was assessed in CTs from nine normal and six IUGR placentae.

3.2.6   [^{125}I]-T$_3$ uptake and efflux assays

T$_3$ uptake and efflux by CTs from normal and IUGR placentae was assessed at 66 hours post-culture when most of the cells had syncytialised. The cells were plated in 12-well plates at a density of 1.5×10^6 cells per well.

3.2.6.1   *T₃ uptake*

After 66 hours in culture, the cells were incubated with 0.5 ml of uptake medium for 0, 5, 10 and 30 minutes in a CO_2 incubator at 37°C. Each time point was performed in duplicate. The uptake medium contained serum free media (SFM) with 1 nM total T$_3$ that consisted of non-labelled T$_3$ and high specific activity [^{125}I]-T$_3$ (Perkin Elmer) so that the final radioactivity added to each well was approximately 200,000 cpm. In order to confirm that the system could be saturated, non-labelled T$_3$ (10 μM) was added to control samples in addition to [^{125}I]-T$_3$. The exact amount of radioactivity added to the cells was determined by measuring the radioactivity in 0.5 ml of uptake medium using a γ-counter (Wallac). At the end of each incubation the uptake medium was removed and the cells were washed quickly three times with ice cold SFM supplemented with 0.1% BSA and were lysed in 2% SDS. The radioactivity in the cell lysates was determined using a γ-counter. T$_3$ uptake was expressed as a percentage of the amount of radioactivity in the cell lysate compared with the amount of radioactivity that was added to the cells. The ability of CTs for T$_3$ uptake was assessed using cells from seven normal and six IUGR placentae.

3.2.6.2   *T₃ efflux*

For assessment of T$_3$ efflux, CTs were incubated with SFM supplemented with 1nM T$_3$ containing [^{125}I]-T$_3$ for 10 minutes in a CO_2 incubator at 37°C. CTs were briefly

washed with SFM with 0.1% BSA and incubated in SFM without T_3 (efflux medium) for 0, 1, 2, 5 or 10 minutes in a CO_2 incubator at 37°C. The medium was then removed and CTs were lysed in 2% SDS. The radioactivity in the cell lysates was determined using a γ-counter. The proportion of radioactivity that was retained in the cell lysate was calculated and compared with the amount of radioactivity that was added to the cells. T_3 efflux was expressed as a percentage of the cellular radioactivity before the addition of efflux medium. T_3 efflux was assessed using cells from six normal and six IUGR placentae.

3.2.7   RNA extraction and quantitative TaqMan PCR

RNA was extracted at 18 and 66 hours post-culture in order to assess gene expression in CTs from normal (n=8) and IUGR (n=8) pregnancies before and after cell syncytialisation. The cells were washed three times with PBS and they were lysed using 1 ml of TRI reagent per well of a six-well plate. The samples were stored at -80°C. Total RNA was later extracted as described in section 2.2.2 and reverse transcribed as described in section 2.2.3. The expression of the TH transporters MCT8, MCT10, LAT1, LAT2, CD98, OATP1A2 and OATP4A1 and of the TR isoforms TRα1, TRα2α and TRβ1 was assessed by quantitative TaqMan PCR following the protocol described in section 2.2.5. TRα1, TRα2 and TRβ1 were amplified using previously validated primers and probes shown in **Table 3-1**. Expression was normalised to the housekeeping gene 18S.

Table 3-1: *Sequences of primers and probes for TaqMan PCR.*

Gene	Forward primer	Reverse primer	Probe	Amplicon length
TRα1	GCTGCAGGCTG TGCTGCTA	CGATCATGCGGAG GTCAGT	CACCTTCATCAGCAGC TTGGGCC	160 bp
TRα2	CAAACACAACA TTCCGCACTTC	GCCCCCTTGTACAG AATCGA	CTCTGCACTTCTCTCT CCTTCATCAGCAGC	80 bp
TRβ1	GTGTCTCAAGTG CCCAGACCTT	CACAGAGCTCGTC CTTGTCTAAGTAA	TGGGGATGTACCCTTT ACATTCTTCTCCTCC	86 bp

All sequences are given in the 5'-3' direction. Underlined base pairs correspond to boundaries between two adjacent exons.

3.2.8 Generation of MCT10 antibody

Rabbit polyclonal antisera for human MCT10 (Ab2198; Charles River; Germany) were raised against a synthetic polypeptide conjugated to keyhole limpet hemocyanine comprising amino acids 503-515 (SSGMFKKESDSII) at the intracellular C terminus of the MCT10 protein. The antisera from final bleeds were affinity purified and their specificity was confirmed by Western immunoblotting using antibody solutions that were pre-incubated with and without the immunising peptide on whole cell protein extracted from CTs or from HTR8 (an EVT-like cell line with low endogenous MCT10 mRNA expression) transfected with either human MCT10 or with VO.

3.2.9 Protein extraction and Western immunoblotting

CTs were lysed at 18 and 66 hours post-culture using 2% SDS and the lysates were stored at -20°C. Protein concentration was assessed using the Bio-Rad Dc protein assay as described in section 2.2.7. Western immunoblotting was performed as described in section 2.2.8. CT lysates (30 μg) were probed with the antibody against MCT8 (1:250) or MCT10 (1:500). MCT8 protein expression was assessed in two separate Western

immunoblots in CTs from six normal and five IUGR placentae. MCT10 protein expression was assessed in CTs from three normal and three IUGR placentae. The expression of β-actin was quantified to assess protein loading. Relative quantification of MCT8 and MCT10 protein expression compared with β-actin was performed by densitometry using the ImageJ software.

3.2.10 Statistical analysis

Data were analysed using the Minitab$^{\circledR}$ statistical software (version 15). Analysis of variance (ANOVA) was performed using the general linear model taking into account all variables that affected the results, such as placental type (IUGR or normal), T_3 treatment and results obtained from the same placental sample, as appropriate. Tukey all pairwise multiple comparisons post-hoc tests were used to assess differences between individual groups. Residuals for all data sets passed the normality test as determined using the Kolmogorov-Smirnov test, with the exception of the hCG secretion data that required log transformation prior to analysis.

3.3   Results

3.3.1   Validation of methods

3.3.1.1   Cell purity

The purity of the primary cultures was regularly assessed by immunofluorescent staining for cytokeratin-7 and vimentin. Representative pictures of cells stained for cytokeratin-7 and vimentin are shown in **Figure 3-3**. There were consistently >95% cytokeratin-7 positive cells and <5% vimentin positive cells.
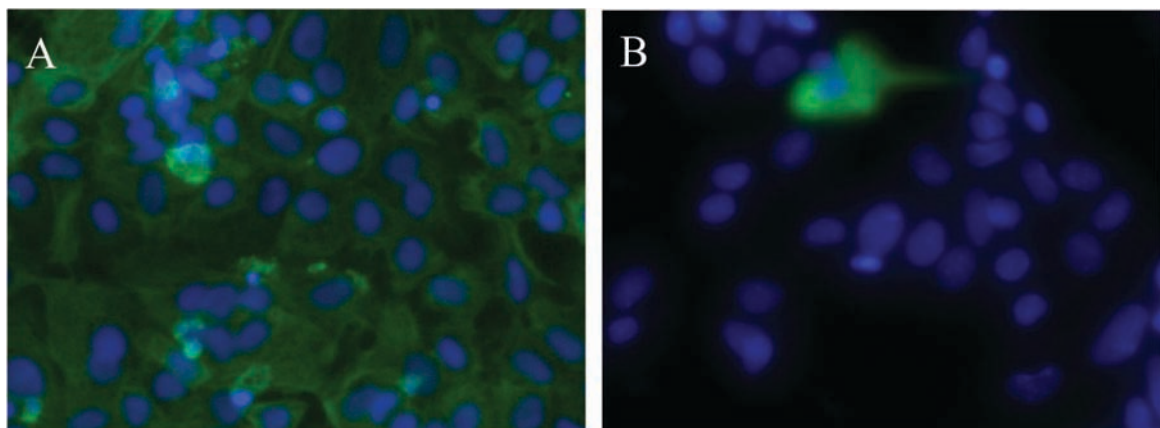
Figure 3-3: *Assessment of cell purity. **(A)** Representative picture of cells stained for the trophoblast marker, cytokeratin-7. The nuclei (blue) were stained using Hoescht 33258 stain. An antibody against cytokeratin-7 was used followed by a secondary anti-mouse antibody labelled with green-fluorescent Alexa Fluor 488 dye. **(B)** Representative picture of cells stained for vimentin. Nuclei are shown in blue (Hoescht 33258 stain). Vimentin protein was localised by indirect staining using an antibody against vimentin and green-fluorescent secondary antibody as described for figure A. All photographed with a 20× objective lens.*

3.3.1.2   Cell syncytialisation

Cell syncytialisation was confirmed by immunofluorescent staining for desmosomes and by quantification of hCG secretion. Representative picture of cells stained with anti-desmosomal antibody at 18 and 66 hours post-culture can be seen in

Figure 3-4A. Secretion of hCG at 18 and 66 hours post-culture was routinely assessed. Results from a representative experiment are shown in **Figure 3-4B**. The secretion of hCG was assessed at 18, 42, 66 and 90 hours post-culture and it was increased 1.3-fold at 42 hours, 5-fold at 66 hours (P<0.01) and 20-fold at 90 hours (P<0.001) compared with 18 hours.
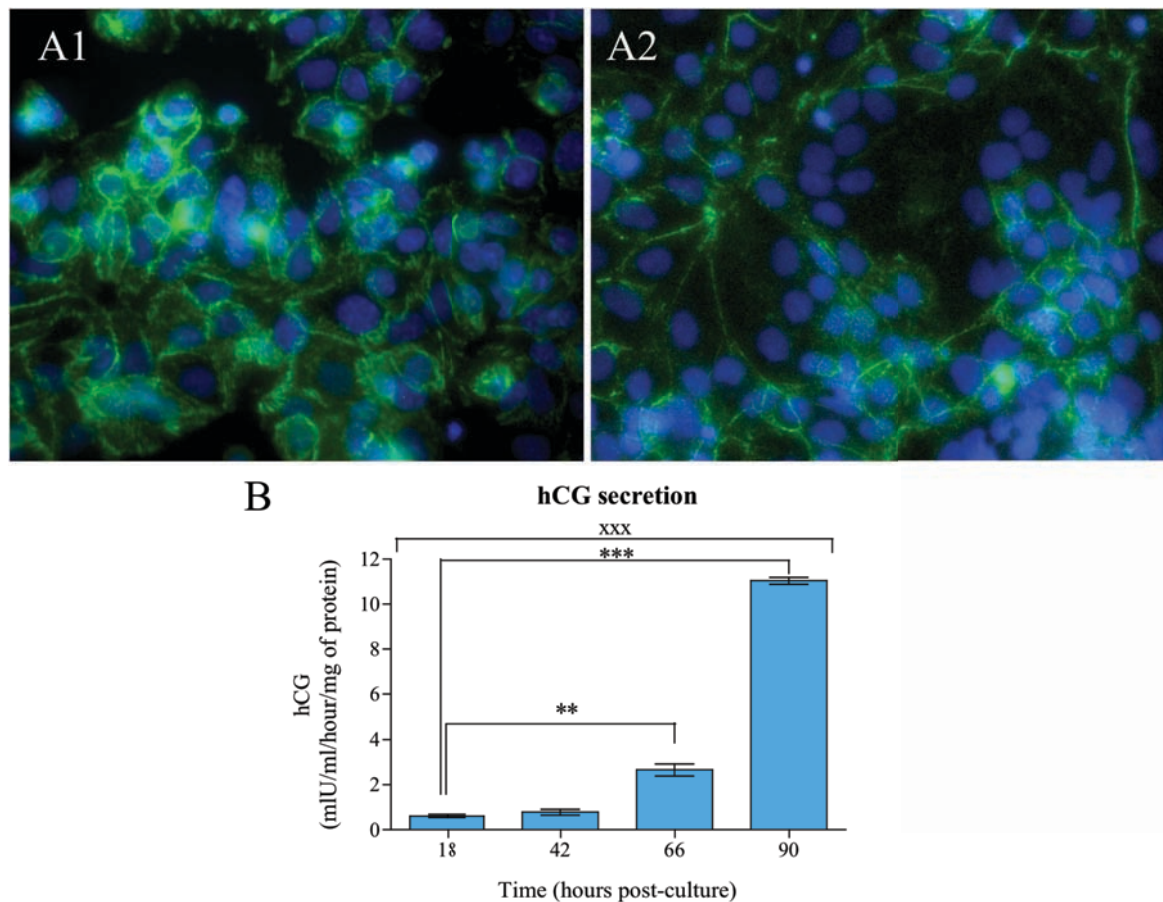
*Figure 3-4: Assessment of cell syncytialisation. (A) Representative pictures showing immunofluorescent staining for desmosomes at 18 (A1) and 66 (A2) hours post-culture. The nuclei, shown in blue, were visualised using Hoescht 33258 stain. Desmosomes were visualised using an antibody against desmosomal protein followed by a secondary anti-mouse antibody labelled with green-fluorescent Alexa Fluor 488 dye. All photographed with a 20× objective lens. (B) Assessment of cell syncytialisation using the hCG secretion assay. hCG secretion was quantified at 18, 42, 66 and 90 hours post-culture using a commercially available ELISA. Bars represent means ±SEM of one experiment performed in triplicate. Overall ANOVA results are indicated by xxx P<0.001. Statistically significant differences between groups are indicated by ** (P<0.01) and *** (P<0.001).*

3.3.1.3   Validation of methods for the assessment of cell survival and apoptosis

The suitability of the MTT assay for the assessment of cell survival was assessed using increasing doses of H_2O_2 to induce cell death by apoptosis. Treatment with H_2O_2 reduced cell viability in a dose-dependent manner as expected **(Figure 3-5A)**. Similarly, H_2O_2 was used for the validation of the methods used for the quantification of the number of cells undergoing apoptosis. Treatment with increasing doses of H_2O_2 increased caspase 3 and 7 activity in the cells, as shown in **Figure 3-5B**. A similar effect was observed when apoptosis in response to H_2O_2 was assessed by immunofluorescent staining for M30 **(Figure 3-5C)**. Representative pictures of cells stained with the M30 antibody are shown in **Figure 3-5D**.
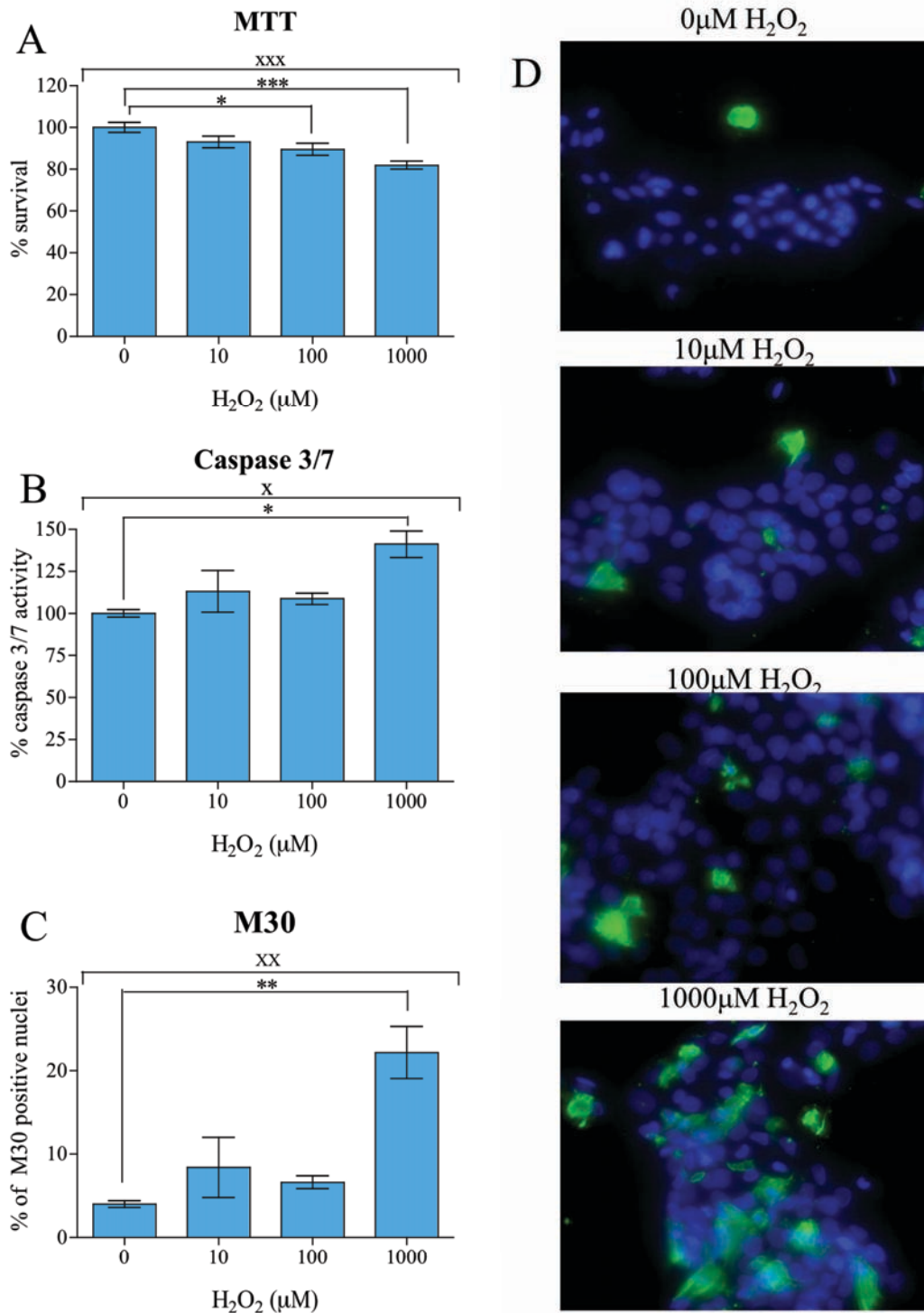
Figure 3-5: *Validation of methods to detect survival and apoptosis of cultured CTs.* ***(A)*** *Following treatment with increasing doses of H_2O_2 for 48 hours cell survival was assessed using the MTT assay in one experiment including four replicates. Apoptosis was assessed using a caspase 3/7 activity assay **(B)** and by immunofluorescent staining for the apoptotic marker M30 **(C)**; both experiments were performed once in triplicate. Bars represent means ±SEM. Overall ANOVA results are indicated by x (P<0.05), xx (P<0.01) or xxx (P<0.001). Statistically significant differences between groups are indicated by * (P<0.05), ** (P<0.01) and *** (P<0.001). **(D)** Representative pictures of cells stained for M30 are shown. The M30 epitope was visualised by indirect staining with an antibody against M30. Nuclei stained with Hoescht 33258 stain are shown in blue.*

3.3.1.4 Validation of the MCT10 antibody

The specificity of the MCT10 antibody was tested using lysates of CTs that had been cultured for 18 hours and lysates of the EVT-like cell line, HTR8, transfected with VO or with MCT10 as controls. MCT10 was detected as a band that migrated at approximately 50 kDa **(Figure 3-6)**. This band disappeared following pre-incubation of the antibody with the blocking peptide and was not present in the negative control sample (HTR8 transfected with VO). Non-specific bands of a higher molecular weight were also observed, however they were also present following pre-incubation with the blocking peptide thus confirming that they were not specific for MCT10.
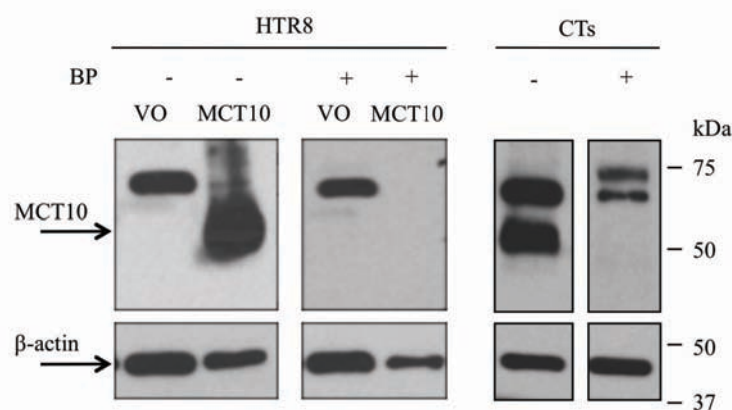
Figure 3-6: *Validation of the MCT10 antibody. The specificity of the MCT10 2198 polyclonal antibody was tested using MCT10-null HTR8 cells transfected with either VO or MCT10 and lysates of CTs cultured for 18 hours. The samples were incubated with the MCT10 antibody pre-incubated with or without the blocking peptide (BP). Immunoreactivity for β-actin was used to assess protein loading.*

3.3.2 Clinical characteristics of study groups

Comparing the demographic data of the normal and IUGR cohorts, there was no significant difference in the maternal age, proportion of cigarette smokers, parity and fetal sex **(Table 3-2)**. However, the median gestational age of delivery in the IUGR cohort was one week less than in the normal (P<0.001). As expected, the birth weights and placental

weights of the IUGR group were significantly lower (P<0.001 and P<0.01, respectively) compared with the normal group. The birth weight percentile, which was calculated using customized growth charts and accounts for parity, ethnicity, maternal BMI, gestational age and fetal sex, was also significantly lower in the IUGR group (P<0.001) with a median of 2nd percentile compared with 53rd percentile for the normal group.

Table 3-2: Clinical characteristics of normal and IUGR study groups.

	Normal (n = 27)	IUGR (n=14)	P value
MATERNAL DATA			
Age (years)	32.0 (41-21)	31.5 (44-24)	NS
Smokers	3/27 (11%)	4/14 (28%)	NS
Nulliparous	3/27 (11%)	5/14 (36%)	NS
FETAL DATA			
Male	9/27 (33%)	5/14 (36%)	NS
Gestational age (weeks)	39 (40-37)	38 (35-40)	0.0006
Birth weight (g)	3320 (2320-4400)	2400 (1565-2960)	<0.0001
Placenta weight (g)	640 (509-883)	464 (313-700)	0.0012
Customized birth weight percentile	53 (23-99)	2 (0-27)	<0.0001
Abnormal Umbilical Doppler Flow	0/27 (0%)	3/14 (21%)	0.0341
Oligohydramnios	0/27 (0%)	8/14 (57%)	<0.0001

Values represent ratios (%) or medians (range). NS = not statistically significant. In the IUGR group, (i) one neonate was diagnosed with Prader-Willi syndrome at eight months of age, (ii) one woman was epileptic treated with a low dose of Lamotrigine (100mg twice daily) and (iii) another woman was a heroin addict who had been taking reducing doses of methadone during pregnancy.

3.3.3 Decreased survival of IUGR CTs in response to T_3 treatment

The effect of T_3 on the survival of normal and IUGR CTs was assessed. Although T_3 treatment did not affect the survival of normal CTs **(Figure 3-7A)**, it significantly

decreased the survival of IUGR CTs (**Figure 3-7B**; P<0.01). Analysis by Tukey multiple

comparison tests revealed that IUGR CT survival was significantly decreased when the

cells were treated with 1 or 100 nM T_3 (14%; P<0.05 and 18% reduction; P<0.01,

respectively). Overall, IUGR CTs survived less compared with normal CTs in response to

T_3 treatment (**Figure 3-7C**; P<0.001). Post-hoc analysis revealed that the difference in

survival between normal and IUGR CTs was more pronounced when the cells were

treated with 1 nM T_3 (20% reduction; P<0.01).
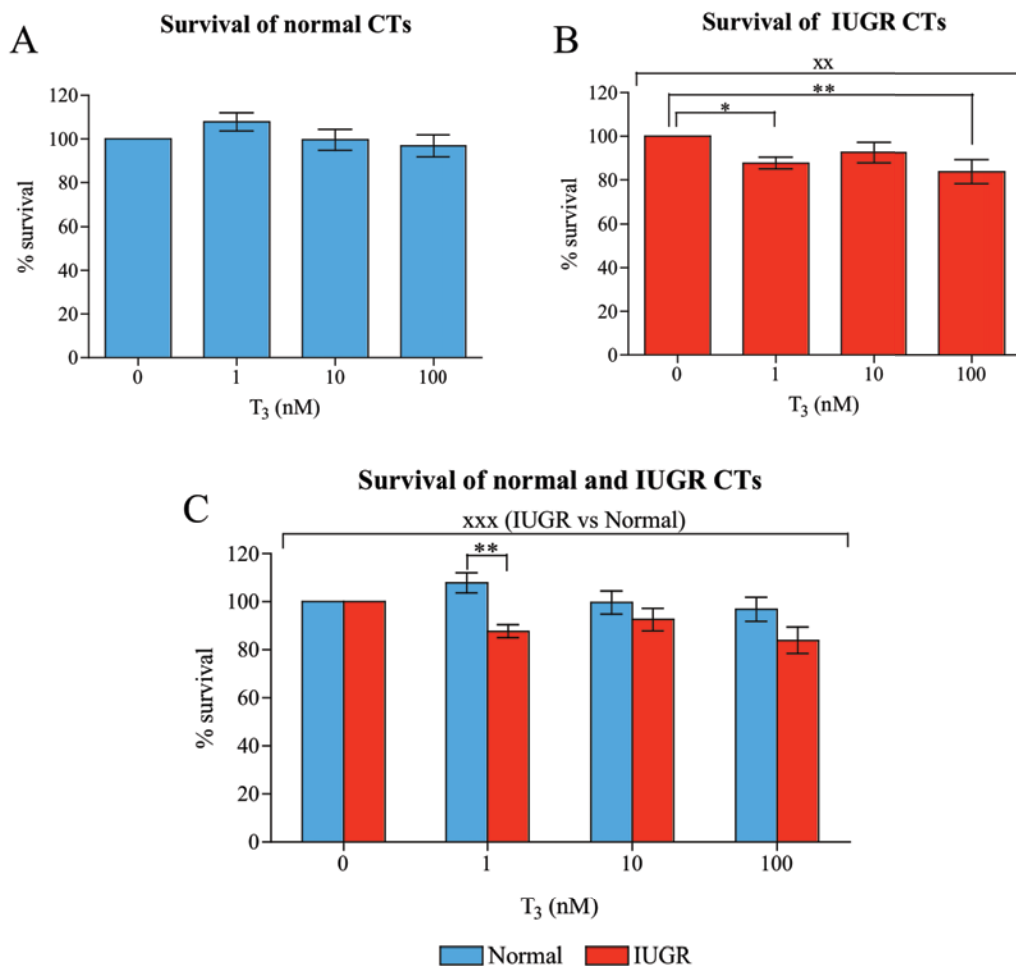
Figure 3-7: *Effect of T_3 on CT survival. CT survival was assessed using the MTT assay 48 hours after treatment with T_3. Cell survival within each experiment is compared with the survival following treatment with 0 nM T_3. (A) Normal CTs, (B) IUGR CTs, (C) Comparison of the survival of normal and IUGR CTs in response to T_3. Overall ANOVA results are indicated by xx (P<0.01) or xxx (P<0.001). Statistically significant differences between groups are indicated by * (P<0.05) and ** (P<0.01). (Normal n=9; IUGR n=5). Bars represent means ±SEM*

3.3.4 Higher apoptosis in IUGR CTs in response to T₃ treatment

The effect of T_3 on cell survival was investigated further by studying apoptosis in normal and IUGR CTs in response to T_3 treatment. Apoptosis was initially assessed using an assay that measures the activity of the effector caspases 3 and 7. T_3 treatment did not affect caspase 3/7 activity in normal CTs **(Figure 3-8A)**. However, in IUGR CTs, T_3 treatment caused an increase in caspase 3/7 activity (**Figure 3-8B**; $P<0.05$). The effect was most prominent when the cells were treated with 100 nM T_3 ($P<0.05$). Overall, caspase 3/7 activity following T_3 treatment was higher in IUGR compared with normal CTs (**Figure 3-8C**; $P<0.001$). Analysis by Tukey post-tests revealed that the difference in caspase activity between normal and IUGR CTs was more pronounced when the cells were treated with 1 or 100 nM T_3 (30% increase for both; $P<0.01$).

This result was confirmed by immunofluorescent staining for the apoptotic marker M30. The percentage of M30 positive nuclei was not affected by T_3 treatment in normal CTs **(Figure 3-9A)**. In IUGR CTs, although there was an increasing trend in the percentage of M30 positive nuclei with T_3 treatment, this effect was not statistically significant **(Figure 3-9B)**. However, it was confirmed that apoptosis is increased in IUGR CTs in response to T_3 treatment compared with normal CTs (**Figure 3-9C**; $P<0.01$), although no statistical differences were found following comparisons between individual groups.
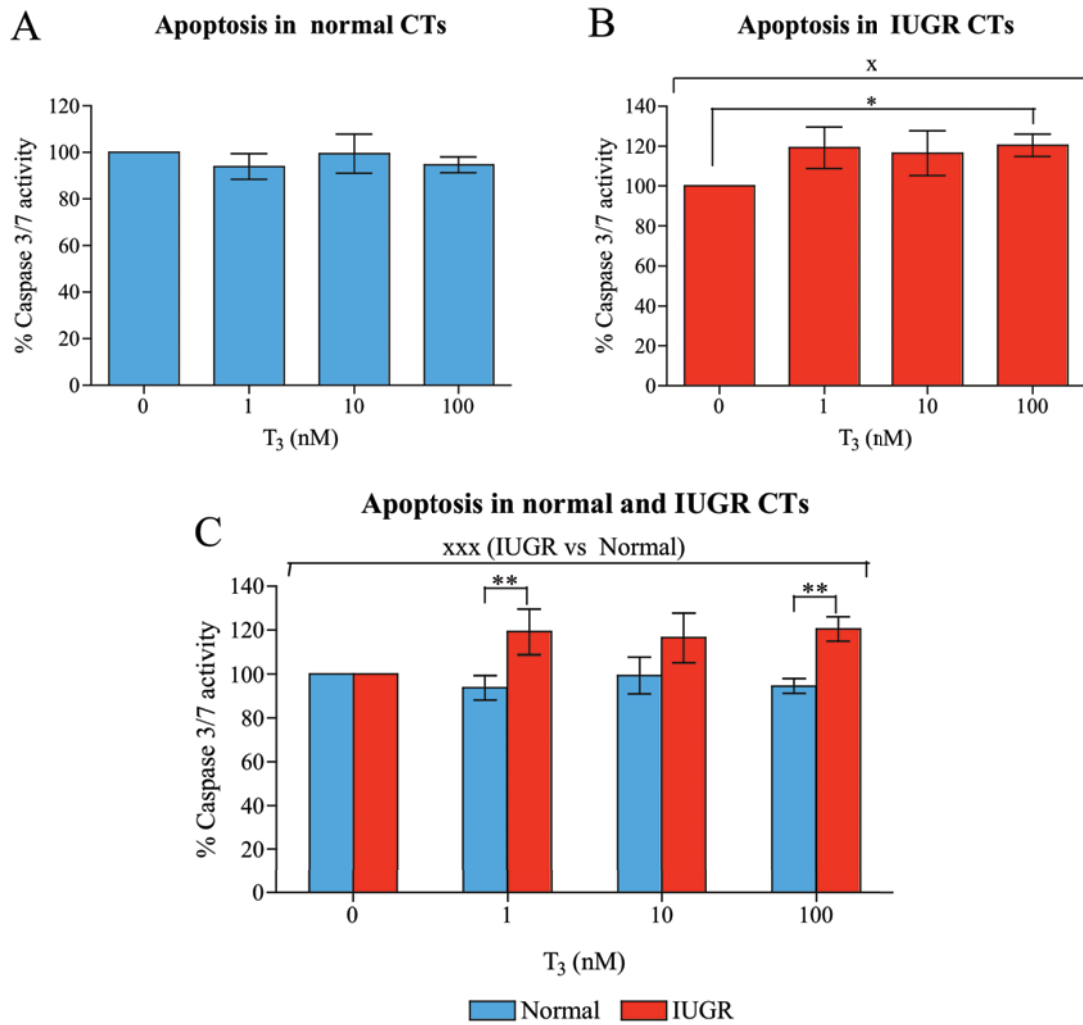
Figure 3-8: *Effect of T_3 on apoptosis using the caspase 3/7 activity assay. Caspase 3/7 activity was assessed 48 hours after treatment with T_3. Caspase activity within each experiment is compared to the activity following treatment with 0 nM T3. (A) Normal CTs (B) IUGR CTs (C) Comparison of apoptosis in normal and IUGR CTs in response to T_3 treatment. Overall ANOVA are indicated by x ($P<0.05$) or xxx ($P<0.001$). Statistically significant differences between groups are indicated by * ($P<0.05$) or ** ($P<0.01$). (Normal n=9; IUGR n=5). Bars represent means ±SEM.*
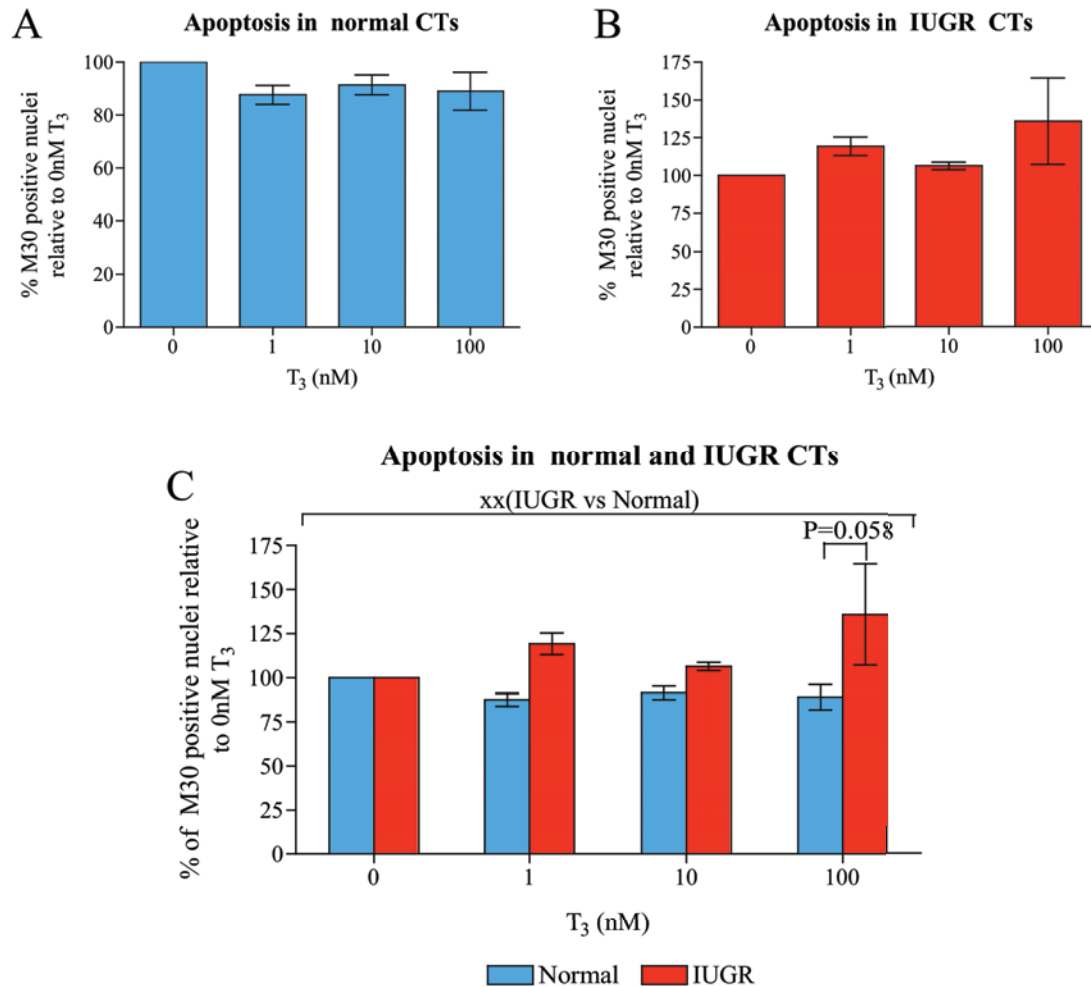
Figure 3-9: *Effect of T_3 on apoptosis using immunofluorescent staining for the apoptotic marker M30. CTs were fixed for immunofluorescent staining 48 hours after treatment with T_3. The percentage of M30 positive cells was calculated for each treatment and it was compared to the percentage of M30 positive cells following treatment with 0 nM T3. (A) Normal CTs (B) IUGR CTs (C) Comparison of M30 immunoreactivity in normal and IUGR CTs in response to T_3. Results following ANOVA are indicated by xx (P<0.01). (Normal n=4; IUGR n=3). Bars represent means ±SEM.*

3.3.5   Effect of T_3 on hCG secretion by IUGR and normal CTs

As shown before, hCG secretion increases with syncytialisation and thus with time post-culture. The rise in hCG secretion was quantified between 18 and 66 hours and between 18 and 90 hours post-culture. Overall, the rise in hCG secretion was higher in

IUGR compared with normal CTs (**Figure 3-10**; Three-way ANOVA: P<0.001). This effect was present both in the absence (P<0.001) and in the presence (P<0.01) of T_3.
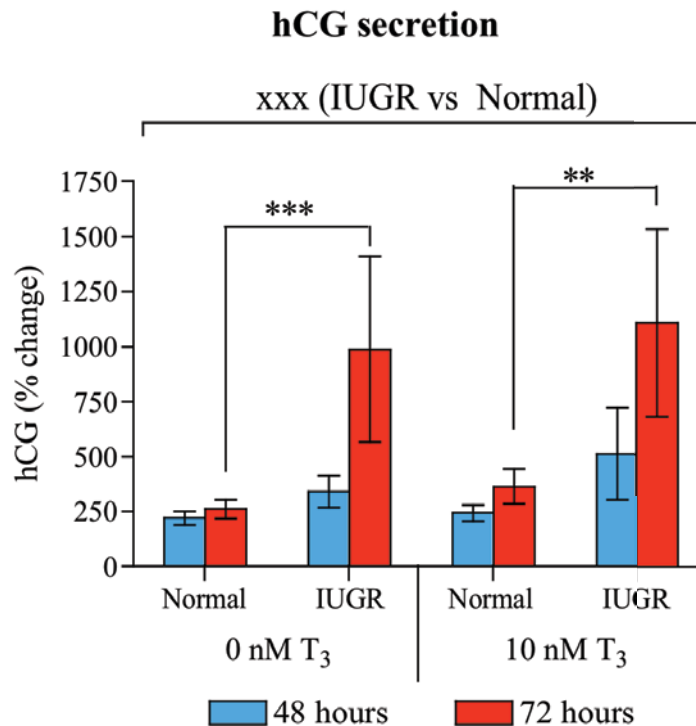
Figure 3-10: *Effect of T_3 on hCG secretion. The rise in hCG secretion was assessed between 18 and 66 hours post-culture and 18 and 90 hours post-culture. Results following analysis by ANOVA are indicated by xxx (P<0.001). Statistically significant differences between groups are indicated by ** (P<0.01) or *** (P<0.001). (Normal n=9; IUGR n=6). Bars represent means ±SEM. Data were log-transformed prior to analysis.*

3.3.6   Increased intracellular accumulation of T_3 in IUGR CTs

The experiments investigating the survival and apoptosis of CTs following T_3 treatment revealed that CTs from normal and IUGR placentae respond differently to T_3. The ability of normal and IUGR CTs for T_3 uptake and efflux was assessed in order to investigate whether the observed difference in T_3 responsiveness could be partly due to differences in intracellular T_3 availability.

T_3 uptake by normal and IUGR CTs was assessed following a time course of incubation with $[^{125}I]$-T_3. Overall, T_3 uptake was higher by IUGR compared with normal CTs ($P<0.001$; **Figure 3-11**). Following incubation with $[^{125}I]$-T_3 for 30 minutes, T_3 uptake by IUGR CTs was 23% higher than uptake by normal CTs ($P<0.001$).
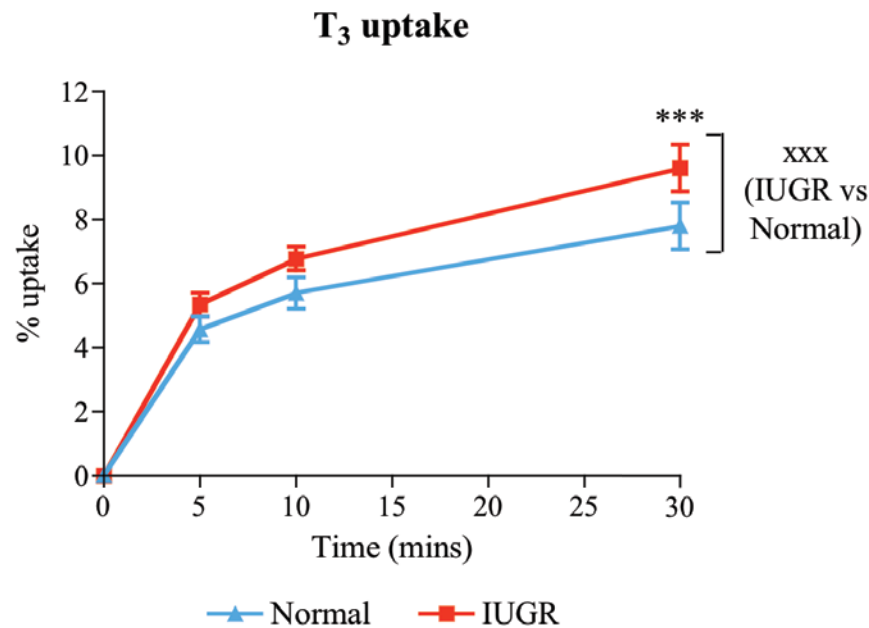
Figure 3-11: *T_3 uptake by CTs from normal and IUGR placentae. CTs were incubated for 0 to 30 minutes with $[^{125}I]$-T_3 (200,000 cpm) and the amount of intracellular radioactivity was assessed. Results following analysis by ANOVA are indicated by xxx ($P<0.001$). Statistically significant differences between normal and IUGR CTs at individual time points are indicated by *** ($P<0.001$). (Normal n=7; IUGR n=6). Points represent means ±SEM.*

Assessment of T_3 efflux by IUGR and normal CTs revealed no differences between the two groups **(Figure 3-12)**. These results therefore suggest that intracellular accumulation of T_3 is higher in IUGR CTs.
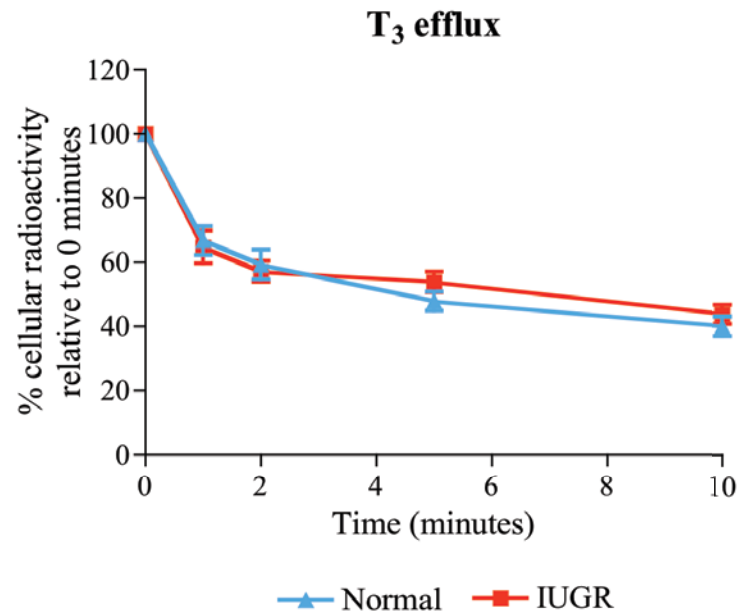
Figure 3-12: *T₃ efflux by CTs from normal and IUGR placentae. CTs were incubated for 10 minutes with [^{125}I]-T₃. After brief washing they were incubated with serum free media without T₃ for 0 to 10 minutes. The amount of radioactivity that was retained intracellularly was assessed. Results following analysis by two-way ANOVA are indicated by x (P<0.05). (Normal n=6; IUGR n=6). Points represent means ±SEM.*

3.3.7   TH transporter expression in IUGR and normal CTs

The expression of the TH transporters in primary cultures of CTs was assessed at 18 and 66 hours post-culture in order to determine whether changes in the expression of TH transporters were associated with the observed changes in TH responsiveness and transport between normal and IUGR CTs. There were no differences in the expression of MCT8 or MCT10 mRNA with IUGR **(Figure 3-13A and B)**. The protein expression of MCT8 and MCT10 was investigated in primary cultures of CTs by Western immunoblotting **(Figure 3-13C1 and D1)**. MCT8 protein expression was increased in

IUGR samples compared with normal samples, in both samples harvested after 18 hours in culture and after 66 hours in culture. Quantification by relative densitometry demonstrated that MCT8 protein expression was increased in IUGR compared with normal CTs (Two-way ANOVA: $P<0.01$) particularly at 18 hours post-culture (2.1-fold increase; $P<0.01$) **(Figure 3-13C2)**. In contrast, there were no changes in the protein expression of MCT10 between normal and IUGR CTs **(Figure 3-13D2)**. In addition, in both normal and IUGR CTs, the expression of MCT8 and MCT10 decreased significantly between 18 and 66 hours post-culture at both the mRNA ($P<0.001$) and protein (MCT8: $P<0.001$; MCT10: $P<0.05$) levels.

Relative quantification of the mRNA encoding the TH transporters LAT1, LAT2, CD98, OATP1A2 and OATP4A1, which have been reported in the human placenta, revealed no significant differences between IUGR and normal CTs **(Figure 3-14)**.
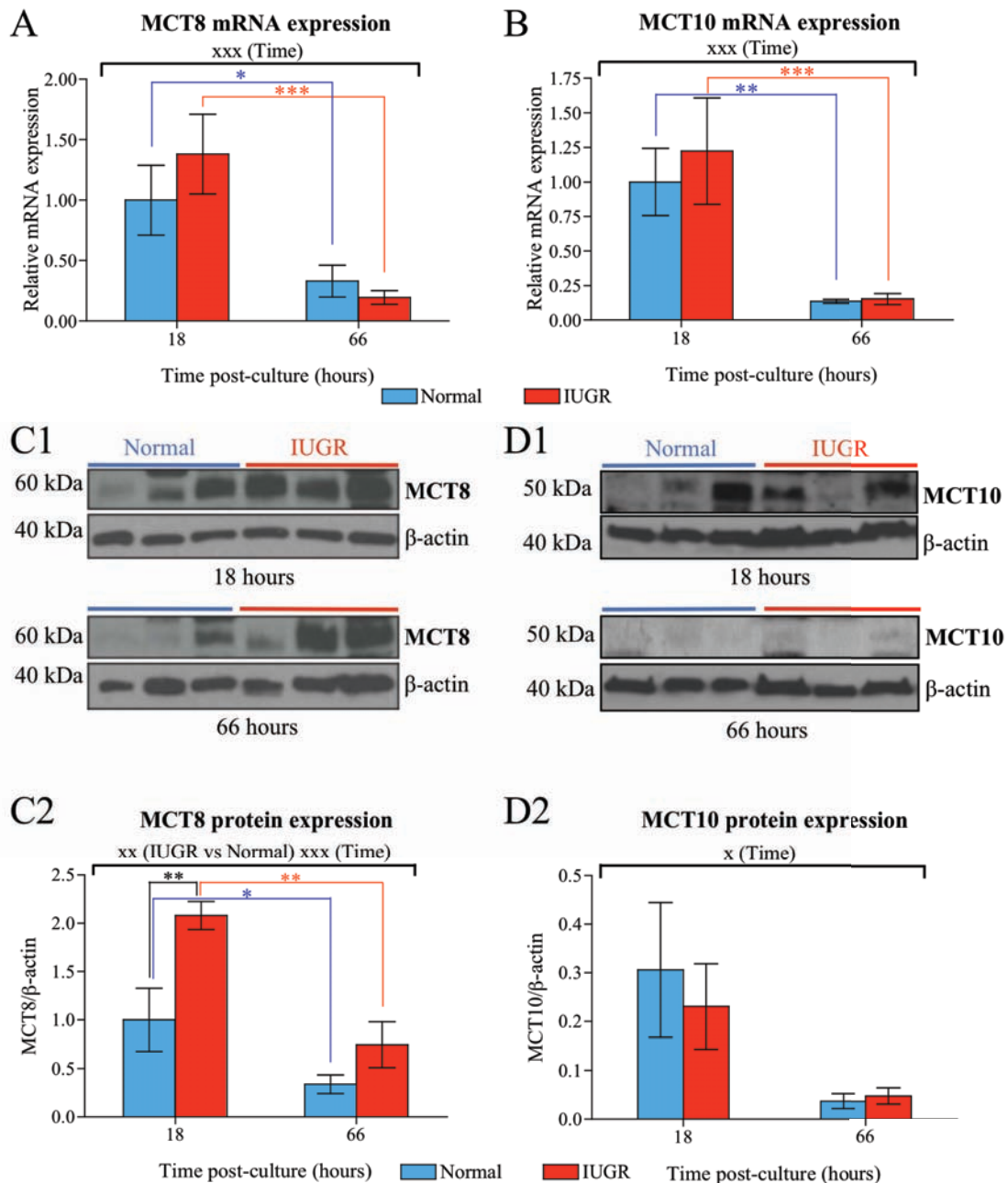
Figure 3-13: *MCT8 and MCT10 expression in primary cultures of normal and IUGR CTs. Relative expression of MCT8 **(A)** and MCT10 **(B)** mRNA (mean +SEM) was assessed at 18 and 66 hours post-culture. Expresion is compared to the mean mRNA expression in normal samples at 18 hours post-culture that was given an arbitrary value of 1. (Normal n=8; IUGR n=8). Representative Western immunoblotting for MCT8 **(C1)** and MCT10 **(D1)** in whole protein lysates obtained from primary cultures of normal and IUGR CTs at 18 and 66 hours post-culture. A band representing MCT8 was detected at approximately 60 kDa. MCT10 was detected at approximately 50 kDa. Immunoblotting for β-actin on the same membranes was used to assess protein loading. The samples shown were obtained from three normal and three IUGR placentae. Changes in the expression of MCT8 (normal n=6; IUGR n=5) **(C2)** and MCT10 (normal n=3; IUGR n=3) **(D2)** protein were quantified by relative densitometry. Expression is shown as a ratio to β-actin protein expression (mean +SEM). Results following analysis by two-way ANOVA are indicated by x (P<0.05), xx (P<0.01) or xxx (P<0.001). Statistically significant differences between groups are indicated by * (P<0.05) ** (P<0.01) or *** (P<0.001).*
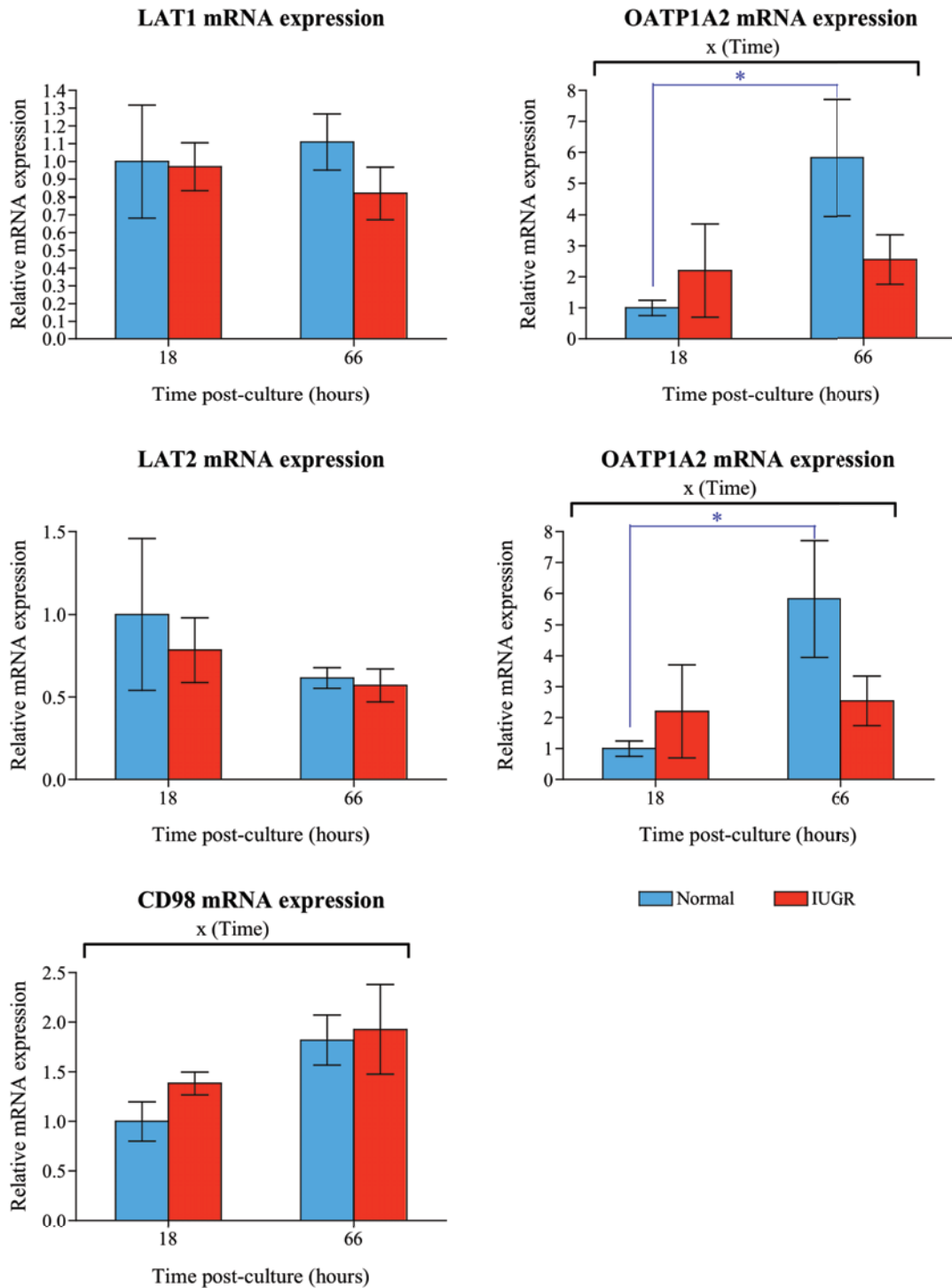
Figure 3-14: *Relative mRNA expression of LAT1, LAT2, CD98, OATP1A2 and OATP4A1 (mean ± SEM) was assessed at 18 and 66 hours post-culture. Expresion is compared to the mean mRNA expression in normal samples at 18 hours post-culture that were given an arbitrary value of 1. Results following analysis by ANOVA are indicated by x (P<0.01). Statistically significant differences between groups are indicated by * (P<0.05) (Normal n=8; IUGR n=8).*

3.3.8   Expression of TRs in CTs from normal and IUGR placentae

It was previously reported that the expression of the TR isoforms TRα1, TRα2 and TRβ1 was increased in villous placental sections from severe IUGR compared with normal pregnancies (Kilby *et al.*, 1998). Increased TR expression may result in increased binding of T$_3$ intracellularly and thus explain the increased T$_3$ responsiveness and increased accumulation of T$_3$ within IUGR CTs. In order to investigate TR expression in primary CT cultures, the mRNA expression of TRα1, TRα2 and TRβ1 was assessed in normal and IUGR CTs that were cultured for 18 or 66 hours **(Figure 3-15)**. There were no statistically significant differences in the mRNA expression of TRs between normal and IUGR CTs. The mRNA expression of TRα2 significantly increased between 18 and 66 hours as assessed by two-way ANOVA (P<0.001). Analysis by Bonferroni post-tests revealed that the increase in TRα2 expression with time in culture was statistically significant within the normal group (4-fold increase; P<0.01). In IUGR CTs, TRα2 mRNA expression increased by 2.5-fold with time in culture but the difference was not statistically significant, whilst there were no statistically significant differences in the expression of TRα1 or TRβ1 with time.
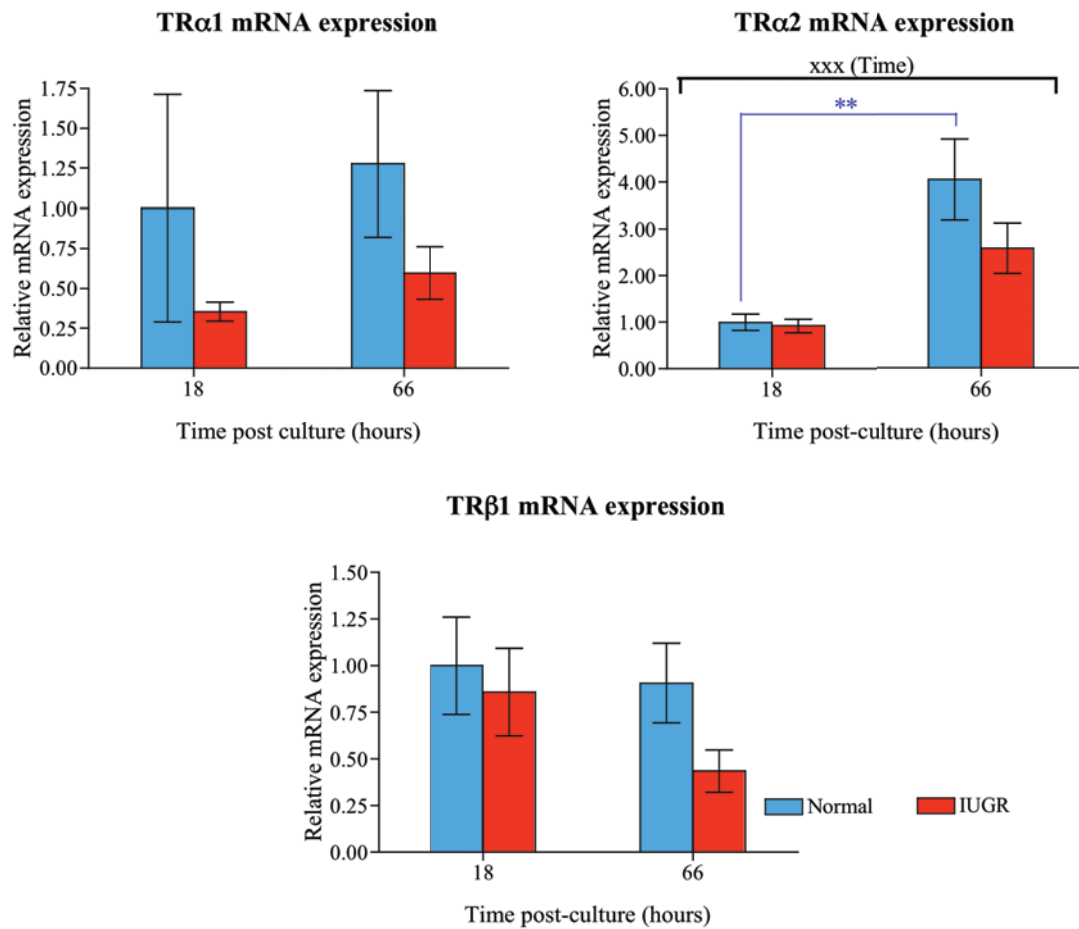
Figure 3-15: *Expression of mRNA encoding TRα1, TRα2 and TRβ1 in primary cultures of normal and IUGR CTs. Relative mRNA expression was assessed at 18 and 66 hours post-culture and compared to the mean expression in normal samples at 18 hours post-culture that were given an arbitrary value of 1. Results following analysis by two-way ANOVA are indicated by xxx (P<0.001). Statistically significant differences in gene expression between groups are indicated by ** (P<0.01). (AGA n=8; IUGR n=8). Bars represent means +SEM.*

3.4   Discussion and conclusions

3.4.1   Differences in T_3 responsiveness of CTs from normal and IUGR placentae

The results described in this chapter demonstrate that CTs from IUGR placentae respond differently to T_3, compared with CTs from normal placentae in terms of cell survival and apoptosis. The findings presented here indicate that T_3 does not affect the survival of normal CTs, which is in accordance with a previous report (Barber *et al.*, 2005). However, T_3 appears to adversely affect the survival of IUGR CTs. Furthermore, there is increased apoptosis in IUGR CTs compared with normal CTs in response to T_3 as demonstrated using a caspase 3/7 activity assay and immunofluorescent staining for M30. These results suggest that increased levels of apoptosis may cause increased cell death of CTs from IUGR placentae in response to T_3 treatment.

It has to be noted that the effect of T_3 treatment on IUGR CT survival and apoptosis was not dose-dependent. Whilst treatment with 1 or 100 nM T_3 decreased the survival and increased apoptosis of IUGR CTs, treatment with 10 nM T_3 did not have a similar effect. In a study by Maruo *et al.*, the authors also described that whilst treatment with 10 nM T_3 induced maximum stimulation of progesterone, oestradiol, hCG and hPL secretion by first trimester human CTs, treatment with either higher or lower concentrations of T_3 gave attenuated effects (Maruo *et al.*, 1991). As discussed in Chapter 1, T_3 exerts its functions by binding to TRs both at the cell membrane and in the cytoplasm leading to the activation of kinase pathways (non genomic action) (Davis and Davis, 1996, Davis *et al.*, 2008), and in the nucleus, where the expression of genes that control cell cycle progression or apoptosis, such as EGF, cyclin D, Mdm2 and p53 are regulated (genomic actions) (Puzianowska-Kuznicka *et al.*, 2006, Yen, 2001). Filipcik *et*

al. investigated the relationship between nuclear TR occupancy and biological effects of T₃ (cell division, TR expression, prolactin secretion and TSH secretion) in the rat pituitary tumour cells, GH4C1 and they reported that the intensity of any particular response is not directly proportional to TR occupancy (Filipcik *et al.*, 1998). In contrast, the induction of a particular biological event requires the occupancy of a different minimal number of nuclear TRs by T₃ and no full receptor population occupancy was required for maximal intensity of the biological events tested apart from TR down-regulation, which was observed following treatment with 20 nM T₃, which corresponds to 100% TR occupancy in these cells. In the case of cell division, occupation of a higher than optimal percentage of TRs inhibited the T₃-stimulated increase of cell division. It is now known that THs can also regulate cell proliferation via the non genomic pathway (Davis and Davis, 1996, Davis *et al.*, 2008). Binding of THs to their cell surface receptor on αvβ3 integrin leads to MAPK activation and translocation to the nucleus, where it induces serine phosphorylation of nuclear TRs and activation of downstream pathways leading to tumour cell proliferation (Davis *et al.*, 2000). In contrast, cytoplasmic TRβ interacts with β-catenin and inhibits the Wnt/β-catenin pathway in thyrocytes resulting in downregulation of cell proliferation (Guigon *et al.*, 2008). Whether the mechanism by which T₃ induces its effects on the survival and apoptosis of IUGR CTs is mediated via the genomic or the non-genomic pathway, or both, is unclear. As indicated by the above examples, it is possible that one pathway promotes while the other inhibits cell survival and that the effect observed is dependent on the balance between the pathways involved. The overall effect of T₃ on CT survival could thus depend on the affinity of the receptors involved for T₃, the number of receptors present and which TRs are expressed in the cells. The possibility for TR downregulation after treatment with high T₃ concentrations, similar to the observations in GH4C1 cells (Filipcik *et al.*, 1998), could further modulate T₃-

mediated effects on cell survival. The lack of dose-dependence in these experiments may thus reflect the activation of separate pathways that are activated and/or saturated by different concentrations of T_3. Future studies are required to determine the pathways that are mediating the effects of T_3 on IUGR CT survival and apoptosis, such as investigating kinase activation and changes in the expression of TH-responsive genes that are involved in apoptosis.

The regulation of apoptosis is important for normal placental development. The ST layer of the placenta is continuously renewed by CTs fusing into the ST layer. Aged STs are being extruded in the maternal circulation as syncytial knots. It is thought that this process is regulated by apoptotic mechanisms (Huppertz and Kingdom, 2004). IUGR has been associated with increased apoptosis in the placenta. Smith *et al* and Ishihara *et al* have demonstrated that there are more apoptotic nuclei in IUGR, compared with normal placentae, particularly in the ST (Ishihara *et al.*, 2002, Smith *et al.*, 1997). Furthermore, the increased level of apoptosis in the IUGR placenta has been previously associated with increased caspase 3 activity (Endo *et al.*, 2005).

Oxidative stress is thought to be one of the factors leading to increased apoptosis in IUGR placenta (Scifres and Nelson, 2009). *In vitro*, altered oxygen tension can cause apoptosis in term placental explants (Heazell *et al.*, 2008). EGF on the other hand, can rescue apoptosis caused by hydrogen peroxide *in vitro* (Moll *et al.*, 2007). It has been shown before that the IUGR placenta is more susceptible to apoptotic stimuli. Crocker *et al* have shown that explants and isolated CTs from pre-eclamptic or IUGR placentae have increased susceptibility to apoptosis induced by exposure to tumour necrosis factor-α or hypoxia compared with normal controls (Crocker *et al.*, 2003, Crocker *et al.*, 2004).

However, this is the first study to show that T_3 can also induce apoptosis in CTs from IUGR placentae *in vitro*, while it has no effect on CTs from normal placentae.

The results presented in this chapter demonstrate that there is increased hCG secretion by IUGR compared with normal CTs, which is in agreement with other studies. Crocker *et al* have assessed hCG secretion and they found that under normoxic conditions (17% oxygen) hCG secretion is higher by explants from pre-eclamptic and IUGR placentae compared with explants from normal placentae (Crocker *et al.*, 2004). Newhouse *et al* found that there was increased syncytialisation in IUGR compared with normal CTs as assessed by immunofluorescent staining for desmoplakin and hCG secretion (Newhouse *et al.*, 2007). The factors that result in increased syncytialisation of IUGR CTs are not known at present. In the experiments presented in this chapter, treatment with 10 nM T_3 did not affect the hCG secretion from either normal CTs or IUGR CTs.

3.4.2 T_3 transport by normal and IUGR CTs

TH transplacental transport is of particular interest since it is critical for normal fetal development (Haddow *et al.*, 1999, Pop *et al.*, 2003, Pop *et al.*, 1999). It has been reported that the serum concentrations of free T_4 and free T_3 are reduced in IUGR fetuses (Kilby *et al.*, 1998). Such changes may arise partly due to inefficient transplacental transport of THs. The changes in the expression of the TH transporters MCT8 and MCT10 in the placenta with IUGR, presented in Chapter 2, suggest that TH transplacental transport may be altered with IUGR. Using primary cultures of CTs from normal and IUGR placentae that were allowed to syncytialise *in vitro*, we observed that net T_3 transport is increased with no change in T_3 efflux by IUGR compared with normal CTs.

These results indicate that there is increased intracellular accumulation of T_3 in IUGR CTs, which may contribute to their increased sensitivity to T_3 treatment.

These results are based on primary cultures of *in vitro* syncytialised CTs that are thought to be representative of STs, the cells that constitute the primary barrier for transplacental transport. However, TH transplacental transport from the maternal to the fetal circulation *in vivo* is more complicated since it involves more cell types, such as endothelial cells and CTs. Therefore, *in vivo* TH needs to be transported across these cells in order to reach the fetal circulation. In this thesis, the transplacental transport of T_3, which is the active TH ligand, was assessed. Even though both T_3 and the prohormone, T_4, are transported across the placenta, T_4 is thought to be the primary TH metabolite for transplacental transport (Chan *et al.*, 2009). Therefore, future studies should also assess transplacental transport of T_4 by normal and IUGR CTs. It is also important to consider that the IUGR placentae collected for this study were from late onset IUGR pregnancies that are less severe than early onset IUGR. However, assessment of T_3 transport in CTs from severe IUGR pregnancies was not feasible due to limited access to such tissues and due to limited availability of gestationally-matched controls.

3.4.3   Changes in the mechanisms that regulate TH availability and action in normal and IUGR CTs

The increased accumulation of T_3 within IUGR CTs may be due to changes in TH transporter expression, changes in T_3-binding capacity within CTs, as well as differences in individual cell volume due to the increased syncytialisation of IUGR CTs.

In the previous chapter, it was reported that the protein expression of MCT8 was increased and the expression of MCT10 was decreased in whole placental biopsies from

pregnancies complicated by severe IUGR compared with gestationally-matched controls, but not in placentae from late onset IUGR pregnancies delivered after 37 weeks. In mononucleate and multinucleate cultures of CTs, even though there were no changes in the mRNA expression of TH transporters, the protein expression of MCT8 was increased with IUGR, whilst there were no changes in the protein expression of MCT10. The discrepancy between the mRNA and protein levels for MCT8 indicates that MCT8 protein levels might be regulated post-transcriptionally, similar to what was observed in whole placenta biopsies (see Chapter 2) and to findings that have been reported for other plasma membrane transporters (Zlender *et al.*, 2009).

The samples used for the primary cultures originated from late onset IUGR pregnancies and gestationally matched normal pregnancies. When we compared the expression of MCT8 between these groups in whole placental homogenates, we found no difference. However, in this case the expression of MCT8 was assessed in purified CTs, therefore the expression pattern might not match the one described in tissue that contains more cell types. The increased protein expression of MCT8 in primary cultures of IUGR CTs may contribute to the increased net T_3 uptake observed by IUGR CTs. Since MCT8 can also facilitate T_3 efflux, our findings suggest that T_3 uptake may be the more dominant role of MCT8 in CTs. No changes were observed in the mRNA or protein expression of MCT10 or in the mRNA expression of LATs or OATPs in IUGR CTs, consistent with previous findings in whole placental biopsies (see Chapter 2). However, the possibility that changes in the activity of these TH transporters may also contribute to the increased intracellular accumulation of T_3 within IUGR CTs cannot be excluded.

It has been previously reported by our group that the protein expression of the TR isoforms TRα1, TRα2 and TRβ1 is increased in the villous placental sections from IUGR

compared with normal pregnancies (Kilby *et al.*, 1998). TRs mediate the action of T_3 by binding to regulatory elements for the DNA and inhibiting or initiating gene expression (Kilby *et al.*, 2005). Therefore, changes in the expression of TRs in IUGR CTs may explain their increased responsiveness to T_3. Furthermore, increased TR expression may result in increased binding of T_3 intracellularly and thus in increased accumulation of T_3 within IUGR CTs. The mRNA expression of TRα1, TRα2 and TRβ1 was assessed in cell lysates from primary cultures and no statistically significant differences were found in the mRNA expression of TRs in IUGR compared with normal CTs. This is in accordance with the study by Kilby *et al.* that similarly did not detect changes in the mRNA expression of TRs, although TR protein was increased in IUGR compared with normal placental tissue (Kilby *et al.*, 1998). Such discrepancies between mRNA and protein expression indicate that TR protein expression may be regulated post-transcriptionally. Assessment of the protein expression of TRs was not possible in these samples due to sample and time limitations. However, these results suggest that assessment of the protein expression of TRs should be assessed in the future to further investigate the argument that changes in TR expression in combination with changes in T_3 transport may lead to altered T_3 responsiveness in CTs from IUGR placentae.

In addition, T_3 action can be locally modulated by the activity of deiodinase enzymes. In a study by Chan *et al.*, it was reported that there were no changes in the activity of deiodinase enzymes D2 and D3 between placental biopsies from IUGR and normal pregnancies (Chan *et al.*, 2003). Furthermore, treatment of normal term CTs with T_3 concentrations within the physiological range (10 nM or less) did not alter the expression of the deiodinase enzymes D2 and D3 in that study. These observations suggest that CTs cannot modulate intracellular T_3 concentration through alterations in D2

and D3 activities, thus rendering IUGR CTs more vulnerable to the increased net T_3 uptake demonstrated in this chapter.

3.4.4 Conclusions

IUGR is multifactorial phenomenon. Maternal hypothyroidism and hyperthyroidism have been implicated in the pathogenesis of IUGR (Abalovich *et al.*, 2002, Casey *et al.*, 2005, LaFranchi *et al.*, 2005, Mestman, 2004). The effects of T_3 on survival and apoptosis of IUGR CTs suggest that abnormal levels of T_3 may contribute to the increased apoptosis observed in placental tissue with IUGR. The differential effect of T_3 on CTs from normal and IUGR placentae may be mediated by the herein reported changes in the expression of the TH transporter MCT8 and by previously reported changes in the expression of TRs with IUGR. However, it is unclear whether the differences in T_3 responsiveness of normal and IUGR CTs are contributing to the aetiology of IUGR or whether they are one of the consequences arising from IUGR. Better understanding of the mechanisms involved in the malfunction of the placenta with IUGR and the possible contribution of T_3 to the pathology of IUGR can help towards the clinical management and potential treatment of IUGR pregnancies.

Chapter 4: The Role of the Thyroid Hormone Transporter MCT8 in Human Primary Cytotrophoblasts

4.1 Introduction

MCT8 has been recently identified as a potent TH transporter (Friesema *et al.*, 2003). Our group has previously reported that MCT8 mRNA is expressed in the human placenta from the sixth week of gestation (the earliest gestation studied) and its expression increases with advancing gestational age (Chan *et al.*, 2006). As discussed in Chapter 2, MCT8 protein is also present in the human placenta from week 8 of gestation until term. Furthermore, MCT8 protein expression is localised in extravillous trophoblasts, villous STs and villous CTs throughout gestation, with more widespread staining at late gestation (Chan *et al.*, 2006). In addition, as discussed in Chapter 2, assessment of the expression of MCT8 in placentae from normal and IUGR pregnancies in the early third trimester and at term has revealed that, although there are no changes in the mRNA expression of MCT8, MCT8 protein is increased with early onset IUGR. This finding was reinforced when MCT8 protein expression was also found to be higher in cultures of CTs obtained from IUGR placentae delivered at term, compared with gestationally-matched normal placentae, as described in Chapter 3.

These findings indicate that MCT8 is important for placental development and strongly suggest that IUGR is associated with changes in the expression of MCT8. These results and the previously discussed effects of T_3 on placental development lead to the hypothesis that MCT8 may affect placental development by mediating the effects of T_3 on trophoblast cells. In the first instance, this hypothesis was tested on primary cultures of CTs obtained from placentae delivered at term. MCT8 expression was silenced or upregulated in the absence or presence of T_3 and effects on (i) cell survival and (ii) hCG secretion were assessed.

4.2   Materials and Methods

4.2.1   Sample collection and CT isolation

This study had approval of the Local Research Ethics Committee (South Birmingham) and the Research and Development Committee of the Birmingham Women's Hospital. Placentae were collected from normal pregnancies following delivery at term by caesarean section. The tissue was processed and CTs were isolated as described in section 3.2.2.

4.2.2   Transfection of human wild-type MCT8

4.2.2.1   Assessment of methods for plasmid transfection

Two different methods were assessed for plasmid transfection of CTs in an initial experiment. The methods tested were transfection using the reagent FuGENE® 6 (Roche) or electroporation using the Amaxa® electroporation method (Lonza, Basel, Switzerland). The CTs were transfected with plasmid encoding green fluorescent protein (pmaxGFP; Lonza) in order to assess and compare transfection efficiency with both methods.

4.2.2.1.1   Plasmid transfection using the FuGENE® 6 reagent

CTs were plated in 35 mm$_2$ dishes (3×10^6 cells per dish), were allowed to adhere overnight and were transfected using FuGENE® 6 on the following day. FuGENE® (6 µl per 35 mm^2 dish) was diluted in serum free medium to a total volume of 100 µl per dish and the solution was incubated at room temperature for 15 minutes. The plasmid (pmaxGFP or VO) was then added to the mix (1 µg per dish) and the solution was

incubated for 20 minutes at room temperature. The mixture was added drop wise to the CTs in each dish and the cells were incubated at 37°C in a CO_2 incubator for 48 hours.

4.2.2.1.2 Plasmid transfection using the Amaxa® electroporation method

The Amaxa® method was used immediately after CT isolation. CTs were permeabilised by electroporation using the Amaxa® Electroporation technique. The CTs were counted and centrifuged at $100 \times$ g for 10 minutes and the cell pellet was resuspended in the provided electroporation solution for primary epithelial cells. The amount of solution used was 100 μl per 3×10^6 CTs. The CTs were transferred to provided cuvettes (3×10^6 cells per cuvette) and plasmid (0.83 μg) encoding GFP was added to each cuvette. CTs were electroporated using five different programs alongside two controls: electroporation in the absence of plasmid DNA or addition of plasmid DNA without electroporation. Following electroporation, CTs were resuspended in CT culture medium at a final concentration of 1.5×10^6 cells per ml and were plated in 35 mm^2 dish at a cell density of 3×10^6 cells per dish.

4.2.2.1.3 Assessment of transfection efficiency and cell recovery

The efficiency of each method was assessed by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, CA, USA) and the data were analysed using the FlowJo software (Tree Star, Ashland, OR, USA). CTs were washed with PBS and trypsinised using 0.25% (w/v) trypsin. They were resuspended in 1 ml media and centrifuged at 1,200 rpm for 10 minutes. The cell pellet was resuspended in 200 μl of 2% (w/v) paraformaldehyde (PFA) and CTs were analysed by flow cytometry in order to identify the proportion of cells that expressed GFP.

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In order to assess cell recovery, the cells were counted under a microscope using a haemocytometer and the number of cells was compared with the number of cells that were not electroporated or incubated with plasmid DNA. As discussed further in the results section, the best transfection efficiency was obtained using the Amaxa® method with electroporation program T-013. Therefore, this method was used in all subsequent experiments.

4.2.2.2   Assessment of expression of transfected MCT8

In order to upregulate the expression of MCT8, CTs were transfected with plasmid (pcDNA3.1+; Invitrogen) containing the human wild-type MCT8 sequence with a HA tag at the C-terminus or with VO as a negative control. The proportion of CTs that expressed transfected HA-tagged MCT8 was assessed by flow cytometry 48 hours after transfection. The cells were washed with PBS and were detached using 15 mM EDTA. They were resuspended in media and centrifuged at 1,200 rpm for 10 minutes. The cell pellet was resuspended in 2% PFA (1 ml) and incubated for 10 minutes at 37°C and then chilled on ice for 1 minute. The cells were centrifuged at 2,000 rpm for 10 minutes at 4°C and the pellet was resuspended in 0.5 ml ice cold 90% methanol. The samples were left on ice for 30 minutes and stored at -20°C until staining.

The cells were washed twice in PBS by centrifugation at 2,000 rpm for 10 minutes and the cell pellet was resuspended in staining buffer (1% FBS in PBS) for 10 minutes in order to block non-specific binding sites. The cells were centrifuged at 2,000 rpm for 5 minutes and were probed with mouse antibody against the HA tag (1:50; Cell Signalling) for 60 minutes in order to detect the transfected MCT8 protein. The cells were rinsed twice in buffer by centrifugation and they were probed with secondary anti-mouse

antibody labelled with green-fluorescent Alexa Fluor 488 dye (1:1,000; Invitrogen) for 30 minutes. The CTs were washed in PBS by centrifugation, resuspended in PBS and analysed by flow cytometry.

MCT8 protein expression was also assessed by Western immunoblotting. CTs were lysed with 2% SDS 66 hours after transfection. Protein concentration was assessed using the Bio-Rad Dc protein assay as described in section 2.2.7. Western immunoblotting was performed as described in section 2.2.8. CT lysates (30 μg) were probed with the antibody against MCT8 (4790; 1:250) followed by secondary mouse HRP-conjugated anti-rabbit antibody (1:2,000; Dako). The expression of β-actin was assessed to control for sample loading.

Transfected MCT8 protein was localised by immunofluorescence. CTs were fixed 66 hours after transfection as described in section 3.2.3 and were probed with rabbit antibody against HA (Santa Cruz; 1:100), followed by secondary anti-rabbit antibody tagged with red-fluorescent Alexa Fluor 594 dye (Invitrogen). Nuclei were stained using Hoescht 33258 stain.

4.2.3   Silencing endogenous MCT8 expression

4.2.3.1   Assessment of methods for transfection with siRNA

Two different methods were initially assessed for the delivery of small interfering RNA (siRNA) into CTs, siPORT™ *NeoFX™* transfection reagent (Ambion) or electroporation using the Amaxa® electroporation method (Lonza). The MCT8 siRNA targeting the third exon of the MCT8 gene was pre-designed and available from Ambion. Non-targeting siRNA (Silencer Negative control; Ambion) was used as a negative control.

The siRNA sequences are shown in **Table 4-1**. The initial experiments carried out to assess the efficiency of siRNA delivery to the cells were performed using the MCT8 siRNA sequence that was tagged with AlexaFluor 488 green-fluorescent dye (Qiagen, West Sussex, UK).

Table 4-1: *Nucleotide sequences for control and MCT8 siRNAs.*

siRNA	Sense sequence	Antisense sequence
Negative control	GGGCCACAGUUUCAGCUUCtt	GAAGCUGAAACUGUGGCCCtt
MCT8	GCCUGCGCUACUUCACCUAtt	UAGGUGAAGUAGCGCAGGCtt

All sequences are given in the 5' – 3' direction.

4.2.3.1.1   *Delivery of siRNA using the siPORT™ NeoFX™ transfection reagent*

CTs were treated with siRNA using the siPORT™ *NeoFX™* reagent immediately after isolation. The siPORT™ reagent was diluted (1:20) in serum free medium (100 μl final volume per 35 mm^2 dish) and incubated at room temperature for 10 minutes. In separate tubes control siRNA and MCT8 green-fluorescent siRNA (0.2 nmoles) were diluted in 100 μl serum free medium. The siPORT™ *NeoFX™* solution and the siRNA solution were combined and incubated for 10 minutes at room temperature to allow transfection complexes to form. The solution was then dispensed in empty tissue culture dishes (200 μl per 35 mm^2 dish). The isolated CTs were diluted at 1.5×10^6 cells per ml. The cell suspension (2 ml) was overlayed on top of the transfection mix and the CTs were allowed to adhere overnight. The final concentration of siRNA in the CT media was 100 nM.

4.2.3.1.2 Delivery of siRNA using the Amaxa® electroporation method

Following CT isolation, CTs were treated with green-fluorescent MCT8 siRNA and permeabilised by electroporation using the Amaxa® electroporation technique. The CTs were counted and centrifuged at $100\times g$ for 10 minutes and the cell pellet was resuspended in the provided electroporation solution for primary epithelial cells. The amount of solution used was 100 µl per 3×10^6 CTs. The CTs were transferred to provided cuvettes (3×10^6 cells per cuvette) containing siRNA (0.2 nmoles) and were electroporated using the program T-013 that was previously determined as the optimal setting (see section 4.2.2.1.3). The cells were resuspended in CT culture medium at a final concentration of 1.5×10^6 cells per ml and 2 ml were dispensed in each 35 mm^2 dish, giving a final siRNA concentration of 100 nM.

4.2.3.2 Assessment of MCT8 siRNA delivery into CTs

In order to assess the efficiency of the siRNA delivery to the cells, the CTs were maintained in culture for 48 hours before they were harvested for analysis by flow cytometry in order to determine the proportion of CTs that contained green-fluorescent siRNA (for protocol see section 4.2.2.1.3).

In order to assess cell recovery the cells were counted using a haemocytometer and the cell number was compared with the number of cells that not electroporated or incubated with siRNA. As discussed further in the results section, the Amaxa® electroporation method was the most efficient method tested for siRNA delivery. Therefore, this method was used in all subsequent experiments.

4.2.3.3   *Assessment of the efficiency of silencing endogenous MCT8*

For all subsequent experiments, endogenous MCT8 was silenced using the Amaxa® electroporation method and using non-tagged MCT8 siRNA.

The silencing of endogenous MCT8 mRNA was assessed by semi-quantitative TaqMan PCR. Total RNA was harvested 24 hours after electroporation as described in section 3.2.7. The RNA was later extracted as described in section 2.2.2 and reverse transcribed as described in section 2.2.3. The expression of MCT8 mRNA was assessed by quantitative TaqMan PCR following the protocol described in section 2.2.5. MCT8 expression was normalised to the expression of the housekeeping gene 18S.

In order to assess the efficiency of MCT8 silencing at the protein level the cells were washed in PBS and lysed with 2% SDS 66 hours after electroporation. Protein concentration was assessed using the Bio-Rad Dc protein assay as described in section 2.2.7. Western immunoblotting was performed as described in section 2.2.8. CT lysates (30 μg) were probed with the antibody against MCT8 (4790; 1:250) followed by secondary goat anti-rabbit HRP-conjugated antibody (1:2,000; Dako). The expression of β-actin was assessed to control for sample loading.

4.2.4   [^{125}I]-T$_3$ uptake assays

T$_3$ uptake was assessed 66 hours after silencing or transfection following the protocol described in section 3.2.6. Due to the limited number of CTs isolated from each placenta, a time course of T$_3$ uptake (0 to 30 minutes) was performed in duplicate within only one experiment. Subsequently, T$_3$ uptake following a 10-minute incubation with [^{125}I]-T$_3$ was assessed in duplicate in four different experiments.

4.2.5   Assessment of cell survival

Following electroporation the cells were seeded in 96-well tissue culture plates at a density of 3×10^5 cells per well in CT media supplemented with 10% SFCS. CTs were allowed to adhere overnight and they were then treated with 0 or 10 nM T_3 18 hours later and maintained in culture for 48 hours. Cell survival was then assessed using the MTT assay as described in section 3.2.5.1. Four replicates were performed for each condition within each experiment. Cell survival was assessed in six separate experiments. For each experiment, absorbance was normalised to the average absorbance detected in CTs transfected with control (control siRNA or VO) and treated with 0 nM T_3.

4.2.6   Assessment of apoptosis

Apoptosis was also assessed 66 hours after electroporation, which is 48 hours after T_3 treatment, using the Caspase 3/7 activity assay as described in section 3.2.5.2.1. Each condition was assessed in triplicate within each experiment. Caspase activity was assessed in six separate experiments. For each experiment, caspase 3/7 activity was normalised to the average activity present in CTs transfected with control (control siRNA or VO) and treated with 0 nM T_3.

4.2.7   Assessment of necrosis

In order to assess whether CT death was occurring via the necrotic pathway a colorimetric assay that measures the activity of lactate dehydrogenase (LDH) released from the cytoplasm of damaged cells in the cell culture media was used (Roche). After electroporation, CTs were allowed to settle overnight in a 96-well plate (3×10^5 cells per well). CTs were treated with 0 or 10 nM T_3 and were maintained in culture for 48 hours.

As a positive control, CTs in three control wells were lysed using 5 μl of provided lysis solution and they were incubated for 15 minutes with shaking. Reaction mixture (100 μl) containing the catalyst (Diaphorase/NAD^+) and a dye solution (Iodotetrazolium chloride and sodium lactate) was added to all wells followed by a 30-minute incubation in the dark. The reaction was stopped by the addition of stop solution (50 μl) to all wells. The absorbance of the samples was measured at 490 nm. Each condition was assessed in triplicate within each experiment. LDH activity was assessed in three separate experiments. Within each experiment, LDH activity for each sample was normalised to the average activity present in CTs transfected with control (control siRNA or VO) and treated with 0 nM T_3.

4.2.8 hCG secretion

The effect on hCG secretion was assessed at 18 and 66 hours after silencing or transfection (0 and 48 hours after T_3 treatment) using the hCG ELISA as described in section 3.2.4.2. The change in hCG secretion between 18 and 66 hours was then calculated. The secretion of hCG was assessed in three separate experiments.

4.2.9 Statistical analysis

Data were analysed using Minitab® statistical software (version 15). When two groups were present, statistical significance was assessed by paired t test. When more than two groups were present, analysis of variance (ANOVA) was performed using the general linear model. Tukey all pairwise multiple comparisons post-hoc tests were used to assess differences between individual groups. Residuals for all data sets passed the normality test as determined using the Kolmogorov-Smirnov test.

4.3   Results

4.3.1   Validation of transfection methods

4.3.1.1   Efficiency of transfection with MCT8

The efficiency of the Amaxa[®] electroporation method and the FuGENE[®] 6 method were assessed by flow cytometry of CTs transfected with pmaxGFP or with VO control. From the Amaxa[®] electroporation programs tested, program T-013 resulted in the best transfection efficiency (53% GFP positive cells) and cell recovery (48%) compared with control. In contrast, the proportion of CTs that expressed GFP following transfection with FuGENE[®] 6 was approximately 3% (**Figure 4-1**; **Table 4-2**). The Amaxa[®] method was therefore chosen as the most efficient method tested.
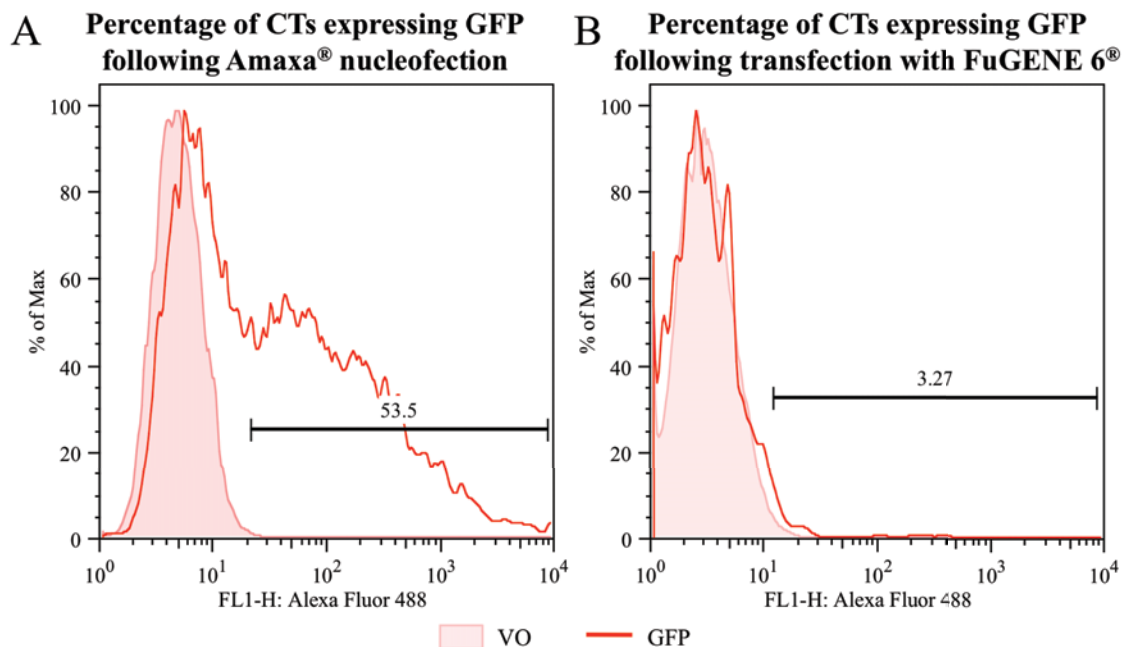
Figure 4-1: *Assessment of the efficiency of plasmid tranfection of CTs. The percentage of cells that were successfully transfected with pmaxGFP by the Amaxa[®] electroporation method **(A)** or using FuGENE[®] **(B)** was assessed by flow cytometry. Cells transfected with VO (pcDNA3.1+) were used as negative control. One representative experiment out of three is shown for Amaxa nucleofection. Transfection efficiency with FuGENE was tested once.*

Table 4-2: *Assessment of cell recovery and efficiency of transfection with GFP using the Amaxa® method or the FuGENE® 6 reagent.*

Transfection method	% Cell recovery compared with control	% GFP-positive cells
Amaxa® electroporation		
Program T-009	15	29
Program X-010	12	42
Program A-020	38	2
Program D-024	36	29
Program T-013	**48**	**53**
No electroporation	28	0
No DNA (Program T-013)	55	0
No electroporation; No DNA control	100	0
FuGENE® 6		
FuGENE:DNA ratio: 6:1	70	3

In order to assess the percentage of cells expressing MCT8 following transfection by Amaxa® electroporation, the proportion of cells that expressed transfected (HA-tagged) MCT8 protein was assessed by flow cytometry. Following transfection with MCT8-HA approximately 28% of the cells were positively stained for HA **(Figure 4-2A)**. Cells transfected with VO were used as negative control. The increase in the expression of MCT8 protein was confirmed by Western immunoblotting using the antibody against MCT8 **(Figure 4-2B)**. MCT8 protein was detected as a band at approximately 60 kDa in cells transfected with VO. A band with much higher intensity was present in cells transfected with MCT8. In addition, transfected MCT8 protein was predominantly localised in the cell membrane as detected by immunofluorescent staining using an

antibody against HA **(Figure 4-2C)**. Cells transfected with VO were used as a negative

control.
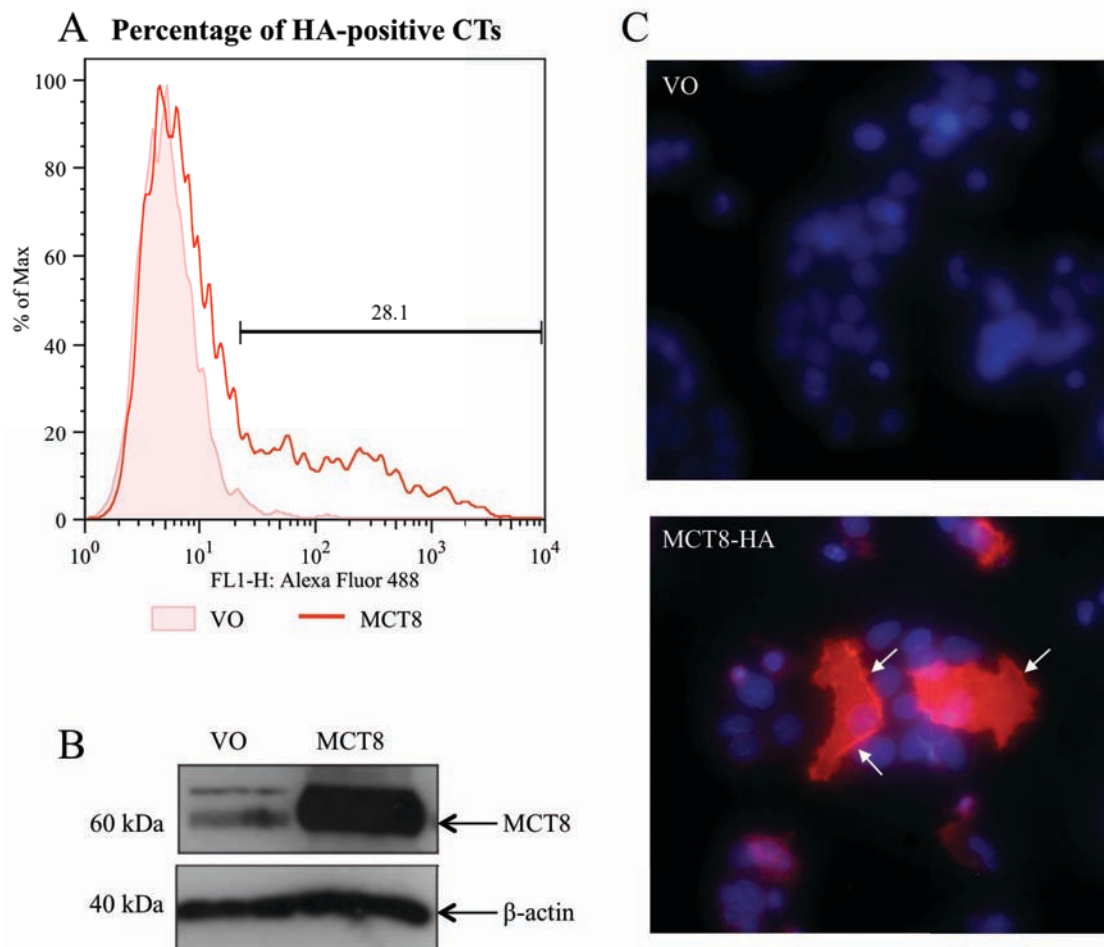
Figure 4-2: *Efficiency of MCT8 transfection. **(A)** The percentage of cells that were successfully transfected with HA-tagged MCT8 was assessed by flow cytometry. The cells were probed with anti-HA antibody followed by secondary antibody labelled with green-fluorescent Alexa Fluor 488 dye. Cells transfected with VO were used as negative control. One representative experiment is shown out of four experiments performed. **(B)** Transfection efficiency was assessed by Western immunoblotting. Lysates of CTs transfected with VO or MCT8-HA were probed with anti-MCT8 antibody followed by secondary anti-rabbit antibody conjugated with HRP **(C)** Localisation of transfected protein at the cell membrane (indicated by arrows) by immunofluorescence. CTs transfected with VO or MCT8-HA were probed with anti-HA antibody followed by secondary antibody labelled with red-fluorescent Alexa Fluor 594 dye. Nuclei are shown in blue. Photographed with a 40× objective lens.*

4.3.1.2   Silencing of endogenous MCT8

The number of cells that were positive for green-fluorescent MCT8 siRNA following transfection using siPORT™ or Amaxa® electroporation was assessed by flow cytometry. Cells transfected with non-fluorescent control siRNA were used as negative control. The proportion of cells positive for fluorescent siRNA using the Amaxa® method was 90% (**Figure 4-3A**; **Table 4-3**). Approximately 21% of the cells were positive for fluorescent siRNA when using the siPORT™ *NeoFX*™ reagent (**Figure 4-3B**; **Table 4-3**). Cell viability was 48% and 80%, respectively **(Table 4-3)**. Therefore, the Amaxa® method was chosen as the most efficient method tested.
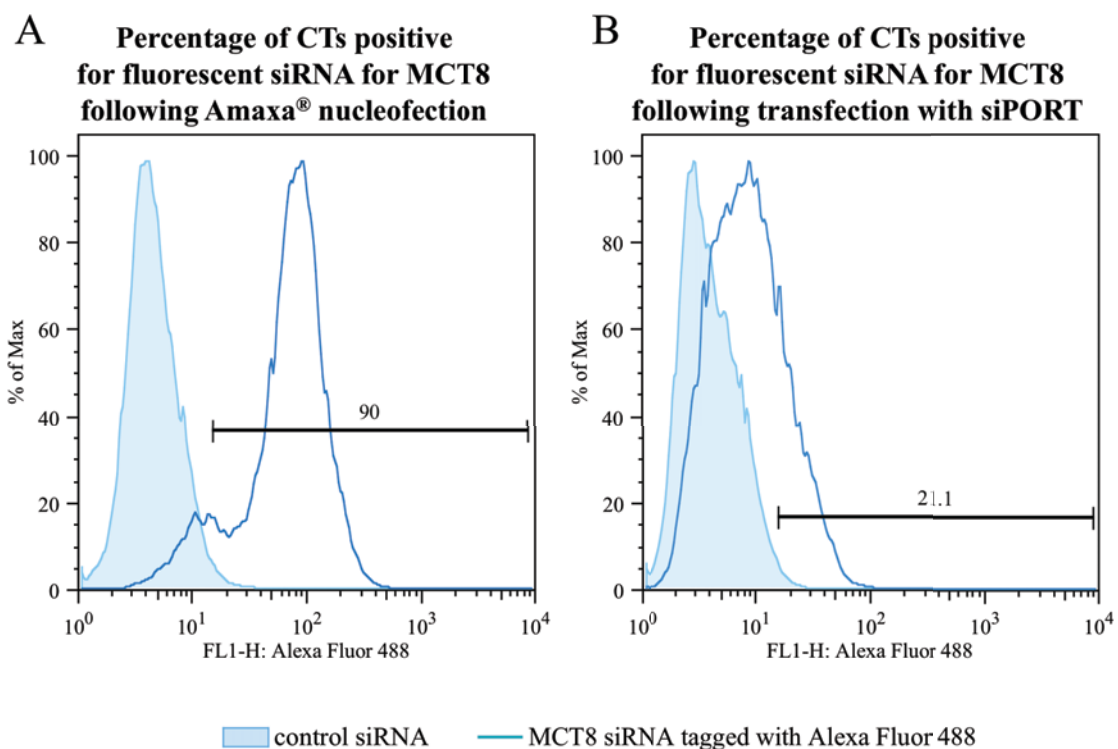
Figure 4-3: *Efficiency of siRNA delivery into CTs. The percentage of cells that were successfully transfected with siRNA by the Amaxa® electroporation method (A) or using siPORT™ (B) was assessed by flow cytometry. Cells were transfected with control siRNA (negative control) or with MCT8 siRNA tagged with Alexa Fluor 488 dye.*

Table 4-3: *Assessment of cell recovery and efficiency of siRNA delivery using the Amaxa®method or the siPORT™ NeoFX™ reagent.*

Transfection method	% cell recovery	% cells with green fluorescent siRNA
Amaxa® program T-013	48	90
siPORT™ *NeoFX™*	80	21

The silencing of MCT8 mRNA was assessed in three different experiments by TaqMan PCR. Endogenous MCT8 mRNA expression was reduced by approximately 90% in the samples treated with MCT8 siRNA compared with the samples treated with control siRNA **(Figure 4-4A)**. In order to confirm that the changes in mRNA expression were translated into changes at protein level, expression of endogenous MCT8 protein was assessed by Western immunoblotting **(Figure 4-4B)**. Endogenous MCT8 was detected as a band at approximately 60 kDa in samples that were treated with control siRNA. No band of that size was present in cells treated with MCT8 siRNA.
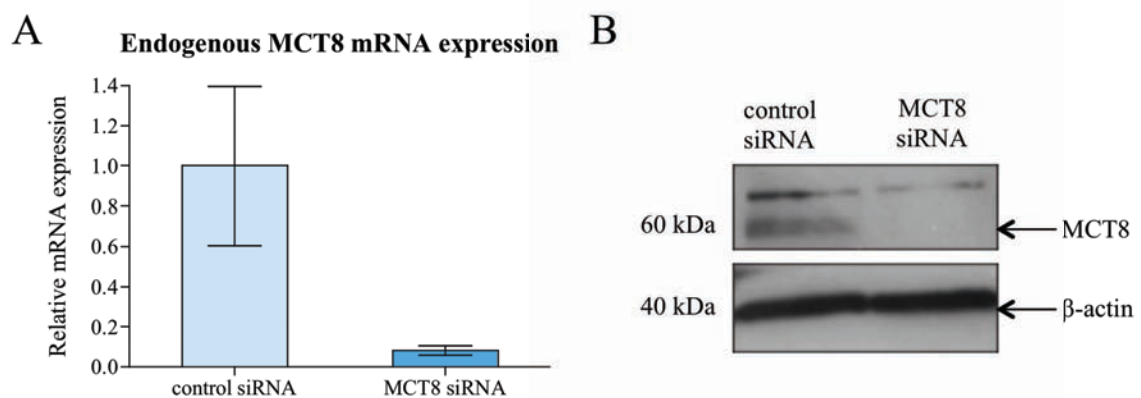
Figure 4-4: *Efficiency of MCT8 silencing. (A) Silencing of endogenous MCT8 mRNA expression was assessed by semi-quantitative TaqMan PCR. Bars represent mean of three experiments ±SEM. MCT8 expression was normalised to the housekeeping control 18S and was compared to the average expression in cells treated with control siRNA that was given the arbitrary value of one. (B) Silencing of endogenous MCT8 protein was assessed by Western immunoblotting. Cell lysates were probed with anti-MCT8 antibody followed by secondary anti-mouse antibody conjugated to HRP.*

4.3.2 T_3 uptake following changes in MCT8 expression

In order to ensure that changes in protein expression of MCT8 following silencing or transfection resulted in functional changes in T_3 uptake, the ability of the cells to uptake T_3 was assessed 66 hours after electroporation. T_3 uptake by transfected cytotrophoblasts was lower than that observed with untransfected cytotrophoblasts (see Chapter 3). This is possibly due to increased death following transfection, resulting in decreased cell number. A time course of T_3 uptake was performed within one experiment, which confirmed that silencing of endogenous MCT8 resulted in a 30% decrease in T_3 uptake following 10 or 30 minutes incubation with $[^{125}I]$-T_3 **(Figure 4-5A1)**. Transfection of MCT8 resulted in a 15% increase in T_3 uptake following 5 or 10 minutes incubation with $[^{125}I]$-T_3 **(Figure 4-5B1)**. However, this difference was not maintained following a 30-minute incubation period.

Due to limitations in the number of CTs obtained following isolation from each placenta, it was not practical to carry out T_3 uptake time courses for more experiments. In order to quantify the changes in T_3 uptake in more experiments, T_3 uptake was assessed following incubation with $[^{125}I]$-T_3 for 10 minutes in separate experiments obtained from four different placentae. Silencing endogenous MCT8 expression resulted in a 20% decrease in T_3 uptake (**Figure 4-5A2**; $P<0.05$) whilst transfection with MCT8 resulted in a 15% increase in T_3 uptake (**Figure 4-5B2**; $P<0.05$).
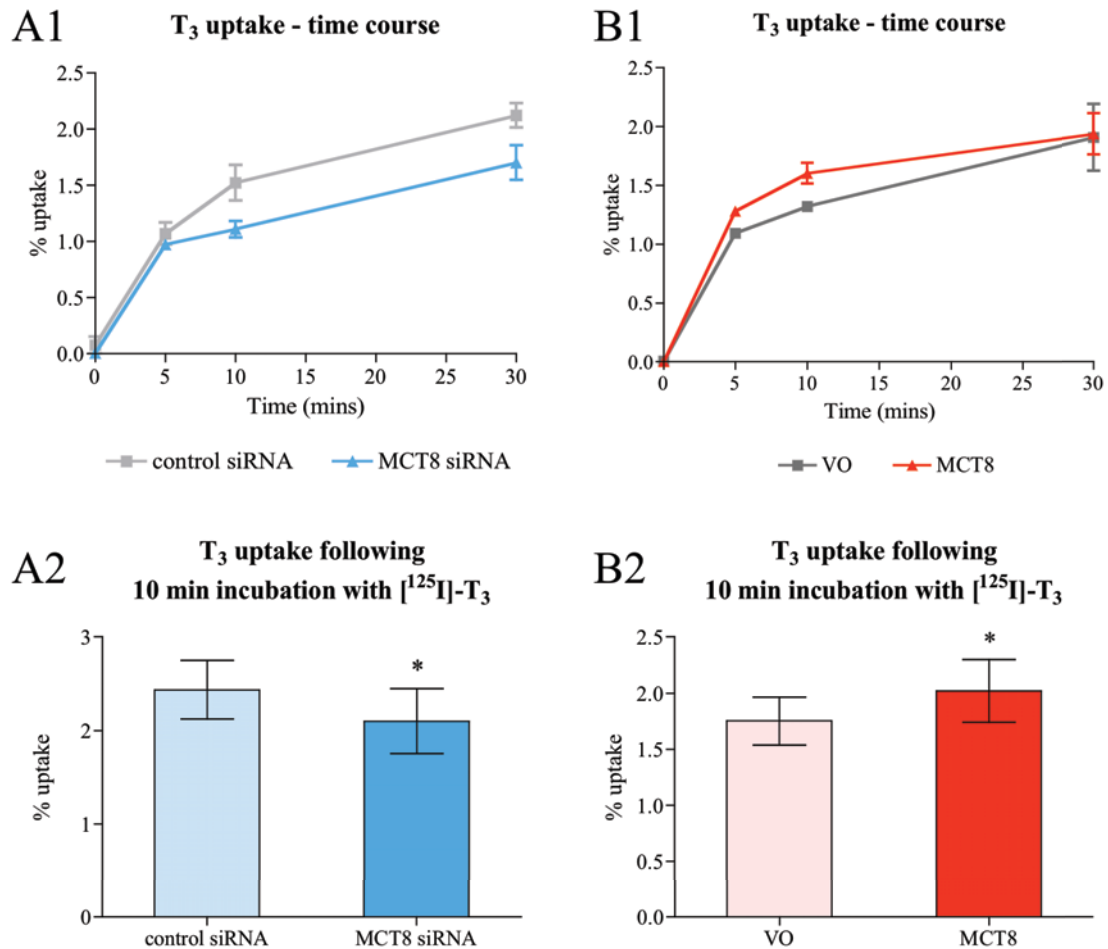
Figure 4-5: *T_3 uptake following MCT8 silencing **(A)** or MCT8 transfection **(B)**. T_3 uptake time course was performed in duplicate in one experiment following silencing of endogenous MCT8 **(A1)** or transfection with MCT8 **(B1)**. CTs were incubated for 0 to 30 minutes with $[^{125}I]$-T_3 and the amount of intracellular radioactivity was assessed. T_3 uptake following incubation for 10 minutes with $[^{125}I]$-T_3 was assessed following MCT8 silencing **(A2)** or transfection with MCT8 **(B2)**. Bars represent mean of four experiments ±SEM. Statistically significant differences were determined by paired t tests and are indicated by * (P<0.05).*

4.3.3   MCT8 adversely affects CT viability

CT viability was assessed following silencing or upregulation of MCT8 expression. Cell viability was increased by approximately 20% following silencing of endogenous MCT8 and treatment with either 0 or 10 nM T_3 (**Figure 4-6A**; P<0.05 for

both). Transfection with MCT8 decreased cell viability by approximately 20% both in the

absence of T_3 (P<0.05) and following treatment with 10 nM T_3 (P<0.01) **(Figure 4-6B)**.
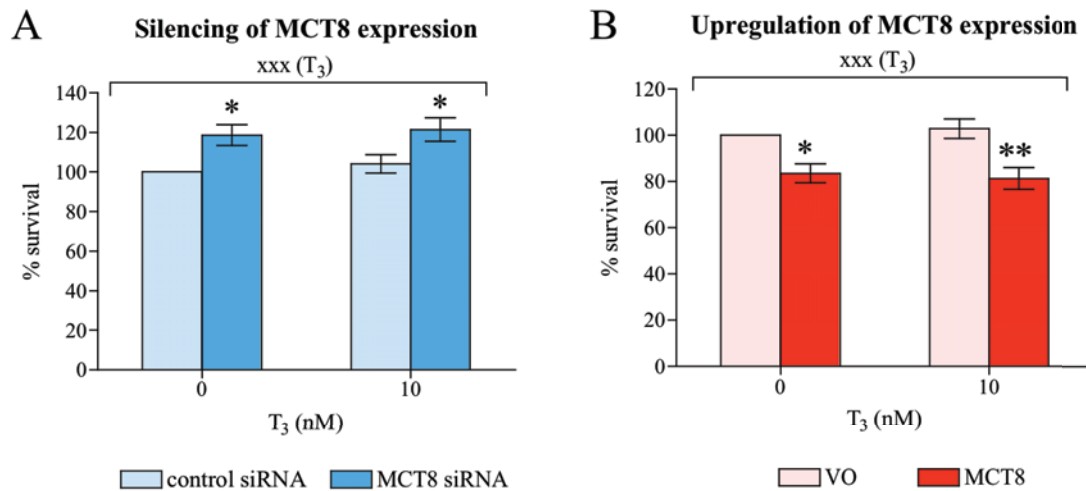
Figure 4-6: *CT survival following silencing of endogenous MCT8* ***(A)*** *or transfection with human wild-type MCT8* ***(B)***. *Survival was assessed by MTT assay 48 hours after T_3 treatment. The absorbance of each sample was normalised to the average absorbance measured in cells treated with control siRNA and 0 nM T_3* ***(A)*** *or in cells transfected with VO and treated with 0 nM T_3* ***(B)***. *The bars represent mean of six experiments ±SEM. Results following analysis by ANOVA are indicated by xxx (P<0.001). Statistically significant differences between groups analysed by Tukey post-tests are indicated by * (P<0.05) and ** (P<0.01).*

4.3.4   MCT8 does not appear to affect apoptosis of CTs

Caspase 3/7 activity levels were assessed as a measure of cell apoptosis. Although

a trend of increased caspase 3/7 activity following silencing of endogenous MCT8 and

decreased caspase 3/7 activity following transfection with MCT8 was observed

independent of T_3 treatment, these differences were not statistically significant (**Figure**

4-7).
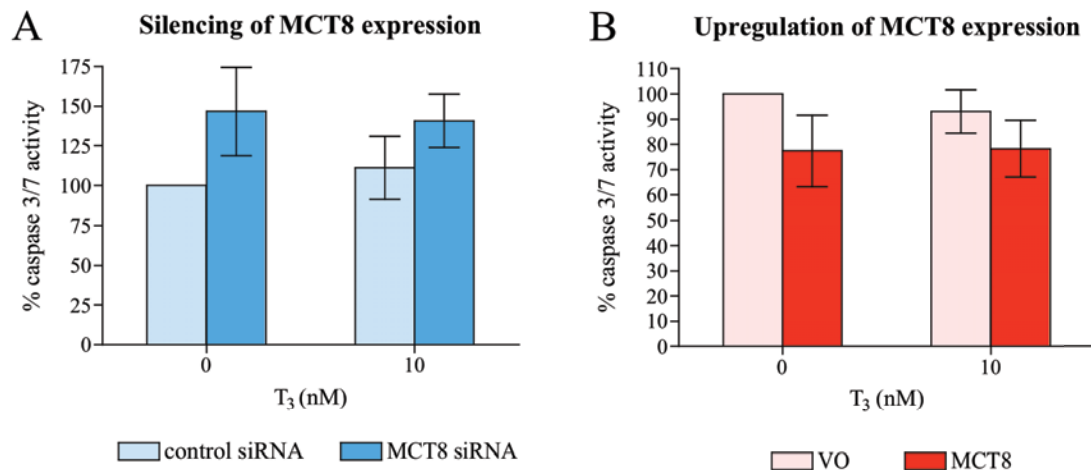
Figure 4-7: *CT apoptosis following silencing of endogenous MCT8 **(A)** or transfection with human wild-type MCT8 **(B)**. Caspase 3/7 activity was assessed 48 hours after T$_3$ treatment as a measure of apoptosis. The caspase 3/7 activity in each sample was normalised to the average activity present in cells treated with control siRNA and 0 nM T$_3$ **(A)** or in cells transfected with VO and treated with 0 nM T$_3$ **(B)**. The bars represent mean of six experiments ±SEM.*

4.3.5   MCT8 does not appear to affect necrosis of CTs

The possibility that cell death occurs via necrosis rather than apoptosis was investigated by assessment of the levels of lactate dehydrogenase activity in the cell culture media. There was no change in cell necrosis following MCT8 silencing or upregulation **(Figure 4-8)**. Cell necrosis was not affected by T$_3$ treatment either.
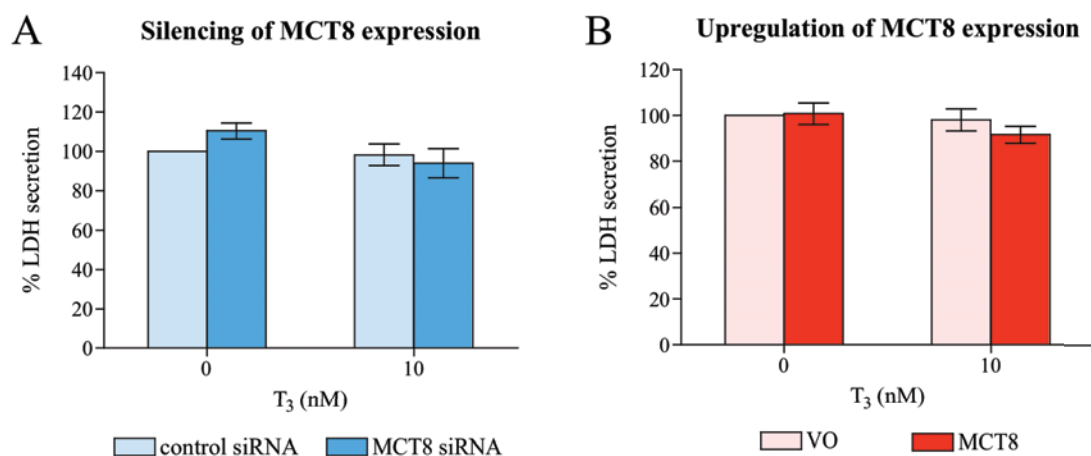
Figure 4-8: *CT necrosis following silencing of endogenous MCT8 **(A)** and transfection of human wild-type MCT8 **(B)**. LDH activity in the cell media 48 hours after T$_3$ treatment was assessed as a measure of cell necrosis. LDH activity in each sample was normalised to the average activity present in cells treated with control siRNA and 0 nM T$_3$ **(A)** or in cells transfected with VO and treated with 0 nM T$_3$ **(B)**. The bars represent mean of three experiments ±SEM.*

4.3.6   MCT8 does not appear to affect hCG secretion

As discussed in Chapter 3, CTs syncytialise *in vitro* to form multinucleate CTs that secrete hCG. The secretion of hCG was assessed following silencing or upregulation of MCT8 expression in the absence or presence of T_3. The secretion of hCG was not affected either by changes on MCT8 expression or by T_3 treatment **(Figure 4-9)**.
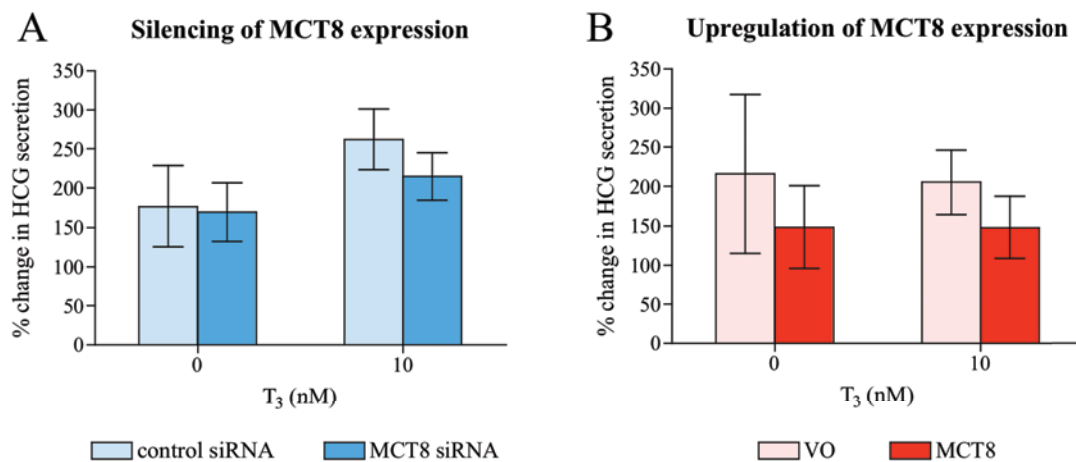
Figure 4-9: *Secretion of hCG by CTs following silencing of endogenous MCT8 **(A)** and transfection with human wild-type MCT8 **(B)**. The bars represent the percentage change in hCG secretion between 18 and 66 hours post-culture. Mean of three experiments ±SEM.*

4.4   Discussion and conclusions

4.4.1   MCT8 impairs CT viability independently of T_3

The results presented in this chapter indicate that MCT8 overexpression impairs the viability of CTs independently of T_3. This is in accordance with previous findings that MCT8 impairs proliferation of the choriocarcinoma cell line, JEG-3, and of the neuronal precursor cell line, N-Tera-2, both in the absence and presence of T_3 (James *et al.*, 2009). The mechanisms by which MCT8 mediates its adverse effect on cell viability and proliferation are presently unknown. The ability of MCT8 to transport compounds other than THs, such as monocarboxylates (lactate) and aromatic amino acids (leucine, tyrosine and tryptophan) has been tested and it has been shown that MCT8 does not mediate the uptake of these compounds (Friesema *et al.*, 2003). The results presented in this chapter raise the possibility that MCT8 transports another compound responsible for the observed effects on cell viability and proliferation, which has to be investigated further. Alternatively, MCT8 may affect the pathways that regulate cell survival and proliferation independently of its transporter function. The possibility that MCT8 may act through T_3-independent mechanisms is further supported by the observations that the phenotype of males affected by MCT8 mutations fail to replicate those found in congenital hypothyroidism (Dumitrescu *et al.*, 2004, Friesema *et al.*, 2004, Schwartz *et al.*, 2005).

The effect of MCT8 on cell apoptosis was assessed in an attempt to identify the mechanisms that mediate the changes in cell viability following altered MCT8 expression. Assessment of the activity of the effector caspases 3 and 7 revealed no differences with changes in MCT8 expression suggesting that the changes in cell survival are not mediated by changes in the level of apoptosis in the CTs. The effect of MCT8 on apoptosis was also

investigated in a study by James *et al* which showed that MCT8 overexpression did not

affect caspase 3 and 7 activity in JEG-3 and N-Tera-2 cells (James *et al.*, 2009), similar to

the results observed here in CTs. The possibility that cell death following MCT8

upregulation occurs by necrosis was also investigated and it was shown that there were no

changes in cell necrosis, as assessed by measurement of LDH activity in the cell media.

CT viability was assessed using the MTT assay, which measures the activity of

mitochondrial reductase enzymes and therefore reflects differences in the number of

living cells. Since primary cultures of CTs are post-mitotic and do not proliferate *in vitro*

any differences in cell number can only be attributed to cell death. Although the activation

of caspase 3 is occurring at the final stages of cell death by apoptosis, cell death can also

occur via caspase-independent pathways (Lockshin and Zakeri, 2002). Therefore, the

possibility that CT cell death is occurring without cell necrosis or apoptosis via caspase

activation has to be considered. In addition, it should also be considered that the

differences observed using the MTT assay might reflect changes in cell metabolic activity

or in mitochondrial number. Counting the number of cells or nuclei following silencing or

upregulation of MCT8 expression in future studies, could better define whether CT death

occurs in response to MCT8 upregulation.

4.4.2   MCT8 does not affect hCG secretion

As discussed in chapter 3, T_3 did not have an effect on the secretion of hCG by

CTs. In this chapter, hCG secretion was assessed following changes in MCT8 expression

and as shown MCT8 silencing or upregulation did not affect hCG secretion either.

Previously, it has been postulated that MCT8 overexpression may suppress proliferation

and induce differentiation of neuronal cells (James *et al.*, 2009). In primary CTs, MCT8

appears to impair cell viability but it does not affect hCG secretion, which correlates with CT syncytialisation.

4.4.3   Changes in MCT8 expression and impact on T_3 uptake

Changes in the expression of MCT8 resulted in limited but consistent changes in the ability of CTs for T_3 uptake. Although, silencing the expression of MCT8 resulted in complete silencing of MCT8 protein expression, as determined by Western immunoblotting, this only resulted in a 20% decrease in T_3 uptake. Similarly, overexpression of MCT8 transiently increased the ability of CTs for T_3 uptake by only 15%. The small magnitude of the changes in T_3 uptake following MCT8 upregulation could be partly attributed to the transfection efficiency, since only 20% of the cells were successfully transfected with MCT8. However, the change in T_3 uptake following MCT8 silencing does not reflect the change in MCT8 expression, since MCT8 siRNA was successfully delivered to 90% of the cells and resulted in undetectable MCT8 protein level, when assessed by Western immunoblotting. As discussed in Chapter 2, six transporters that mediate TH uptake have been identified in the human placenta to date. Apart from MCT8, these also include MCT10 (Park *et al.*, 2005), LAT1 (Okamoto *et al.*, 2002), LAT2 (Kudo and Boyd, 2001), OATP1A2 (Patel *et al.*, 2003) and OATP4A1 (Briz *et al.*, 2003, Patel *et al.*, 2003, Sato *et al.*, 2003). The transporters with the highest affinity for T_3 are the system L (K_m = 0.8 μM (Friesema *et al.*, 2001)), OATP4A1 (Km = 0.9 μM (Fujiwara *et al.*, 2001)) and MCT8 (rat MCT8 Km = 4.0 nM (Friesema *et al.*, 2003); human MCT8 Km = 0.86 μM (Kinne *et al.*, 2009)). Therefore, the high residual T_3 uptake following MCT8 silencing is possibly due to the presence of the above TH transporters that can facilitate T_3 uptake in the absence of MCT8. These results indicate that there is a

redundancy in the TH transport system in the human placenta and suggest that MCT8 is only responsible for a proportion of the T_3 uptake that is occurring by CTs.

It is thought that both T_4 and T_3 are transported across the placenta (Chan *et al.*, 2009). In this thesis, the focus was on the role of MCT8 in the uptake of T_3, which is the active TH ligand. However, the circulating concentration of T_4 is higher than that of T_3 and it is thought that T_4 is the predominant TH that is transported from the maternal to the fetal circulation (Chan *et al.*, 2009). It is therefore essential to assess the role of MCT8 in the transport of T_4 by trophoblast cells in future studies.

4.4.4   Study limitations

In this study, the role of MCT8 in CTs was investigated by siRNA-mediated knockdown of endogenous MCT8 protein and by upregulation of MCT8 expression by plasmid transfection of human wild-type MCT8. In previously published studies, cell lines have been used to investigate the effects of gene knock-down or upregulation, as the transfection of primary cell cultures has proved challenging. The cell lines that were used to investigate CT function (JEG-3, JAR, BeWo) originate from choriocarcinomas. Although using cell lines is associated with ease of use and good transfection efficiencies, there is criticism on the use of cell lines as models of CTs and the use of primary cultures is advocated instead. It has therefore been essential to identify techniques that result in efficient transfection of primary CTs. In this study the Amaxa$^®$ electroporation method was successfully used to transfect primary CTs. As described in the results section, this method was the most efficient of the methods tested for transfection with either siRNA or plasmid vectors. However, it has to be noted that cell viability was impaired following electroporation. Therefore, it would be useful to continue testing new techniques for

transfection of primary cells as they develop in the future. Recently, Forbes *et al* reported on the efficiency of different techniques for the transfection of primary CTs with siRNA and they have identified a reagent (DharmaFECT) that resulted in good transfection efficiency (95%) and good cell viability (85%) (Forbes *et al.*, 2009). Furthermore, in another report published recently, microporation was used for the delivery of siRNA to primary CTs from term placenta with 80-90% transfection efficiency and limited cell death (Vargas *et al.*, 2009).

4.4.5   Conclusions

This is the first study to investigate the effect of MCT8 on primary cultures of CTs. The adverse effect of MCT8 on the cell viability of primary CTs is consistent with the postulation that the increased placental expression of MCT8 in IUGR pregnancies may contribute to the maldevelopment and malfunction of the placenta in IUGR pregnancies. The results described here are similar to the effects previously observed in the choriocarcinoma cell line, JEG-3 and further support the evidence that MCT8 has T_3-independent actions. Further studies are necessary in order to elucidate the mechanisms by which MCT8 affects cell proliferation or viability. Such studies in placental cells are of great importance especially in light of the evidence that placental MCT8 expression is increased in IUGR pregnancies. In addition, such investigations can help to improve our understanding of the importance of MCT8 not only in placental development but also in the development of the CNS as clearly displayed in males with MCT8 mutations.

Chapter 5: The Role of the Thyroid Hormone Transporter MCT8 in the Human Extravillous Trophoblast-like Cell Line, SGHPL-4

5.1   Introduction

In the previous chapter, the role of MCT8 in CT cells isolated from term placentae was investigated. As discussed, MCT8 overexpression decreases CT viability independently of T_3, which is of importance for cell turnover and transplacental transport in late pregnancy. Invasion of EVTs into the maternal myometrium and transformation of the maternal spiral arteries are critical events for normal placentation that are initiated early in pregnancy (Fisher *et al.*, 1989). It is therefore important to also assess the role of MCT8 in placental development in early pregnancy and particularly in EVTs.

The human placenta is TH responsive from early gestation. As discussed in Chapter 2, a range of TH transporters are expressed in the human placenta from week 6 of gestation suggesting that first trimester placental tissue has the capacity for TH uptake. Our group has previously reported that mRNA and protein of the TR isoforms TRα1, TRα2 and TRβ1 are expressed in first trimester human placenta (Kilby *et al.*, 1998) and TRα1, TRα2 and TRβ1 proteins were localised in both endovascular and interstitial EVTs in placental bed biopsies (Barber *et al.*, 2005). Furthermore, deiodinases 2 and 3 are present and functional in first trimester placenta indicating that there is the potential for the regulation of TH action in the placenta in early pregnancy (Chan *et al.*, 2003). Previous reports on the effect of T_3 on invasion by cultured primary EVTs suggest that T_3 increases the invasive potential of EVTs (Oki *et al.*, 2004) and suppresses EVT apoptosis *in vitro* (Laoag-Fernandez *et al.*, 2004).

Our group has previously reported that MCT8 mRNA and protein are detected in the human placenta from gestational week 6 and 8, respectively and MCT8 protein was localised in EVTs, CTs and STs in the first trimester (Chan *et al.*, 2006). MCT8 may thus

play a role in placental development during the first trimester possibly by regulating T_3 availability. The hypothesis that MCT8 may modulate EVT invasion and cell growth was investigated using SGHPL-4s, an immortalised cell line derived from primary EVTs transfected with the early region of Simian virus 40 (Choy *et al.*, 2000). In this study, SGHPL-4s were used to investigate EVT-like cell proliferation, apoptosis and invasion (i) in response to T_3 treatment and (ii) following silencing or upregulation of MCT8 expression in the absence or presence of T_3.

5.2   *Materials and Methods*

5.2.1   Culture of SGHPL-4 cells

The SGHPL-4 cells were kindly provided by Prof G Whitley (St George's University, London, UK). The cells were cultured in F10 Ham media (Sigma-Aldrich) supplemented with 10% (v/v) FCS, 1,000 units/L Penicillin, 0.001% (w/v) Streptomycin and 0.029% (w/v) L-Glutamine (all from Invitrogen). The cells were passaged twice every week at a split ratio of 1:4 and were used up to passage 25. In order to assess whether SGHPL-4s expressed TRs, total mRNA was extracted and reverse transcribed as described previously (sections 2.2.2 and 2.2.3) and the mRNA expression of TRα1, TRα2 and TRβ1 relative to the expression in biopsies from term placenta was assessed by TaqMan PCR as described in section 2.2.5.

5.2.2   T$_3$ treatment

The effect of T$_3$ on SGHPL-4 proliferation and invasive capacity was investigated. Media supplemented with SFCS, which is stripped of THs, was used instead of normal FCS. SGHPL-4s did not survive when plated in tissue culture wells using SFCS-supplemented media. For this reason, the cells were seeded in tissue culture plates in media supplemented with 10% FCS and were allowed to adhere overnight. The medium was then replaced with 10% SFCS supplemented medium and the cells were incubated for 4 hours. Following this washing period, the medium was replaced with fresh 10% SFCS-supplemented medium and the cells were treated with T$_3$ (0, 1, 10 or 100 nM) for 48 hours. In contrast, SGHPL-4 viability was not affected when the cells were seeded on

Matrigel[®] coated cell culture inserts using SFCS-supplemented medium and thus an initial incubation with FCS-supplemented medium was not required for these experiments.

5.2.2.1   Cell invasion through Matrigel[®]

SGHPL-4 invasion following treatment with 0 or 10 nM T_3 was assessed using cell culture inserts for 24-well plates with 8 μm membrane pore size (BD Biosciences, Erembodegem, Belgium) that were coated with 10μl of growth factor-reduced Matrigel[®] matrix (BD Biosciences) diluted in PBS. The Matrigel[®] was allowed to set and the inserts were added into 24-well tissue culture plates that contained 0.8 ml 10% SFCS-supplemented media. SGHPL-4s (5×10^4) were added on top of each insert in a total volume of 0.2 ml 10% SFCS supplemented media (**Figure 5-1**). The cells were allowed to invade through Matrigel[®] by incubating in a tissue culture incubator (37°C, 5% CO_2) under normoxic conditions for 48 hours. The Matrigel[®] and the cells that remained at the top of the membrane were removed and the cells at the bottom of the membrane were stained with Mayer's haematoxylin for 10 minutes, washed quickly in tap water and incubated for 1 minute in Scott's tap water. The filters were incubated in eosin for 5 minutes followed by a quick wash in tap water. The cells were dehydrated by incubating sequentially in 90% and 99% ethanol. The filters were allowed to air-dry, they were detached and mounted in Vectamount (Dako) on microscope slides. The cells were examined using a microscope with a 20× objective and the cells that invaded through Matrigel[®] were counted and the percentage of cell invasion relative to the cells that were not treated with T_3 was determined. Each condition was performed in triplicate within three separate experiments. All the invasion experiments described in this chapter were performed by Dr Laurence Loubière.
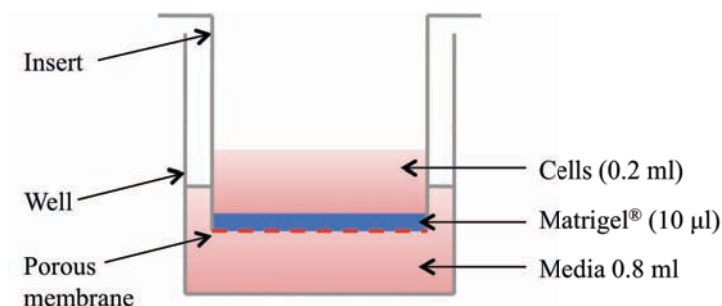
Figure 5-1: *Diagram of cell culture insert set-up for invasion assays.*

5.2.2.2   Cell proliferation and apoptosis

Cell proliferation was assessed 48 hours after T_3 treatment in 96-well plates using the MTT assay as described in section 3.2.5.1. Each condition was repeated four times within four different experiments. Apoptosis was assessed in white-walled 96-well plates 48 hours after T_3 treatment by determining caspase 3/7 activity as described in section 3.2.5.2.1. Each condition was performed in triplicate within four different experiments.

5.2.2.3   mRNA expression assays

The cells were lysed at 0, 2, 6, 16, 24 and 48 hours after T_3 treatment with TRI reagent (Sigma-Aldrich) and total mRNA was extracted as described in section 2.2.2 and reverse transcribed as described in section 2.2.3. Each treatment was performed in duplicate within four different experiments. The mRNA expression of the TH responsive genes Malic Enzyme 1 (ME1) and Connexin 43 (Cx43) in each sample was assessed in duplicate by quantitative TaqMan PCR as described in section 2.2.5 using primers and probes that were previously designed and validated (**Table 5-1**). Expression was normalised to the housekeeping gene 18S.

A diagram of the timeline of T_3 treatment and the subsequent experiments can be seen in **Figure 5-2**.

Table 5-1: *Primer and probe sequences for quantification of ME1 and Cx43 mRNA expression by TaqMan PCR.*

Gene	Forward Primer	Reverse Primer	Probe	Amplicon length (bp)
ME1 (NM_002395)	TTCGGAAGCCAAG AGGTCTCT	CACAATGGCCTT GATGACATCT	TTACTATCCACGATCG AGGGCATATTGCTTC	97
Cx43 (NM_000165)	TTCATTTTACTTC ATCCTCCAAGGA	CGCTCCAGTCAC CCATGTT	CCTGGGCACCACTCTTT TGCTTAAAAGTAGTG	98

All sequences are given in the 5'-3' direction. Underlined base pairs correspond to boundaries between two adjacent exons.
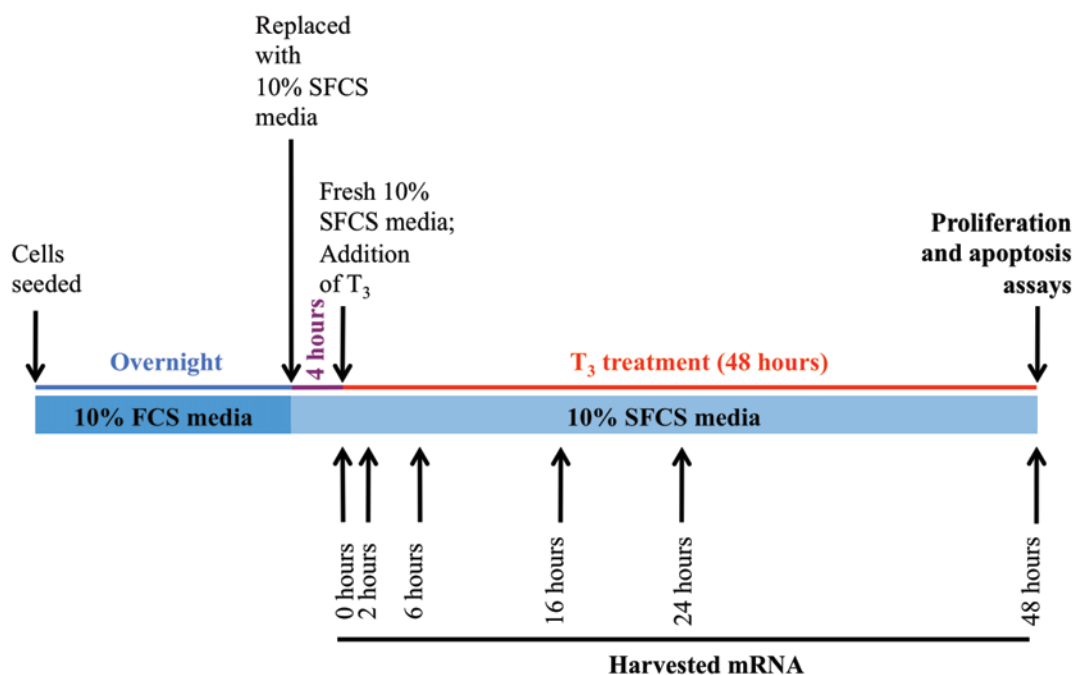
Figure 5-2: *Diagram of T_3 treatment experiments. SGHPL-4s were incubated in 10% FCS supplemented media in tissue culture plates overnight. The media was replaced with 10% SFCS supplemented media. After a 4-hour washing period the media was replaced with fresh 10% SFCS media containing 0,1,10 or 100 nM T_3. The cells were lysed for mRNA extraction at 0, 2, 6, 16, 24 and 48 hours after T_3 treatment. Cell proliferation and apoptosis assays were performed 48 hours after T_3 treatment.*

5.2.3   Silencing and upregulation of MCT8 expression in SGHPL-4s

5.2.3.1   Optimisation of transfection

The SGHPL-4 cells were transfected using the Amaxa$^®$ electroporation method (Lonza) following the protocol described in section 4.2.2.1.2. Initially the cell line optimisation kit provided by the manufacturer was used to identify the optimal solution and electroporation program for the transfection of SGHPL-4s. Cells were harvested by trypsinisation when they were 70-80% confluent and were transfected (2×10^6 cells per cuvette) with plasmid encoding GFP (pmaxGFP) by electroporation using a selection of seven different programs and two different suspension solutions. Cells that were electroporated in the absence of plasmid DNA and cells that were not electroporated were used as negative controls. The percentage of cells that were successfully transfected and were expressing GFP was assessed by flow cytometry 24 hours after electroporation as described in section 4.2.2.1.3. Furthermore, the cells were counted under a microscope using a haemocytometer in order to assess cell recovery compared with cells that were not electroporated or incubated with plasmid DNA. As described further in the results section, the condition that resulted in the greatest transfection efficiency and cell viability (solution V; program X-001) was identified and used for the transfection of SGHPL-4s in subsequent experiments.

5.2.3.2   Transfection of human wild-type MCT8

MCT8 expression was upregulated by transfection with plasmid (pcDNA3.1+) containing the human wild-type MCT8 sequence with a HA tag attached at the C-terminus or with VO as a negative control. SGHPL-4s (2×10^6 cells per cuvette) were resuspended in solution V, transferred to cuvettes containing 2 μg of plasmid DNA and

electroporated using program X-001. Following electroporation, the cells were resuspended in 10% FCS-supplemented media at a final concentration of 2.25×10^5 cells per ml. The proportion of the cells that expressed transfected HA-tagged MCT8 72 hours after electroporation was assessed by flow cytometry using an antibody against the HA tag as described in section 4.2.2.2. MCT8 protein expression was also assessed by Western immunoblotting. SGHPL-4 cells were lysed with 2% SDS 72 hours after transfection with MCT8 or VO. Western immunoblotting was performed as described in section 2.2.8. Cell lysates (70 µg) were probed with the antibody against MCT8 (4790; 1:250) followed by secondary anti-rabbit HRP-conjugated antibody (1:2,000; Dako). The expression of β-actin was used to assess protein loading. Transfected MCT8 protein was localised by immunofluorescence as described in section 3.2.3. SGHPL-4s were fixed 72 hours after transfection and they were probed with antibody against HA (1:100; Cell Signalling) followed by secondary anti-mouse antibody tagged with green-fluorescent Alexa Fluor 488 dye (Invitrogen). Nuclei were stained using Hoechst 33258 stain.

5.2.3.3   Silencing endogenous MCT8 expression

Endogenous MCT8 expression was silenced by transfection with siRNA targeting MCT8 or with non-targeting siRNA as negative control as described in section 4.2.3.1. SGHPL-4s (2×10^6 cells per cuvette) were resuspended in solution V, transferred to cuvettes containing 0.45 nmoles siRNA and electroporated using program X-001. They were resuspended in 10% FCS-supplemented media at a final concentration of 2.25×10^5 cells per ml, giving a final siRNA concentration of 50 nM.

The efficiency of the silencing of endogenous MCT8 mRNA expression in SGHPL-4s was assessed by quantitative TaqMan PCR. The cells were lysed with TRI

reagent (Sigma-Aldrich) 24 hours after transfection. Total mRNA was extracted and reverse transcribed as described previously (sections 2.2.2 and 2.2.3) and the expression of MCT8 mRNA was assessed in extracts from two separate experiments. MCT8 protein expression was assessed by Western immunoblotting. The cells were lysed with 2% SDS 72 hours after electroporation and Western immunoblotting was performed as described in section 2.2.8. SGHPL-4 cell lysates (70 µg) were probed either with the 4790 antibody against MCT8 (1:250), which targets the N-terminus of MCT8, or with the 1306 antibody against MCT8 (1:100; gift from Prof T. Visser), which targets the C-terminus. The blots were then probed with secondary anti-rabbit HRP-conjugated antibody (1:2,000; Dako). The expression of β-actin was used to assess protein loading.

5.2.3.4 $[^{125}I]$-T_3 uptake assays

In order to assess the effect of MCT8 silencing or upregulation on T_3 transport, SGHPL-4s were transfected with MCT8 siRNA or with plasmid encoding human MCT8. In addition, SGHPL-4s were co-transfected with CRYM, a cytosolic protein that binds to T_3 intracellularly and thus prevents T_3 efflux. Cells that were transfected with control siRNA, VO or CRYM alone were used as controls. T_3 uptake was assessed 72 hours after transfection. SGHPL-4s were incubated with $[^{125}I]$-T_3 for 0, 2, 5, 10 and 30 minutes and the proportion of radioactivity that was retained intracellularly was determined as described in section 3.2.6. Each condition was performed in duplicate within three different experiments.

5.2.3.5   *Assessment of SGHPL-4 invasion through Matrigel*®

Following transfection with siRNA (MCT8 siRNA or control siRNA) or plasmid DNA (VO or MCT8) by Amaxa® electroporation the cells were cultured on top of

Matrigel® in 10% SFCS-supplemented medium and were treated with 0 or 10 nM T_3. Cell invasion through Matrigel® was assessed 48 hours later as described in section 5.2.2.1. Each condition was performed in triplicate in five (MCT8 silencing) or eight (MCT8 upregulation) separate experiments and the percentage of cells that invaded relative to controls (cells transfected with control siRNA or VO and treated with 0 nM T_3) was determined.

5.2.3.6 *Assessment of cell proliferation, apoptosis and necrosis*

Following transfection with siRNA or plasmid DNA by Amaxa® electroporation, the cells were plated in 96-well plates and were allowed to adhere during an overnight incubation in 10% FCS-supplemented media. The media was replaced with 10% SFCS-supplemented media and after a 4-hour washing period, the media was replaced with fresh 10% SFCS-supplemented media containing 0 or 10 nM T_3. The cells were cultured in this media for 48 hours, which is 72 hours post-transfection, and cell proliferation, apoptosis and necrosis were assessed. Cell proliferation was assessed using the MTT assay as previously described (section 3.2.5.1). Four replicates were performed for each condition within five different experiments. The caspase 3/7 activity assay was used to assess apoptosis as described in section 3.2.5.2.1. Each condition was assessed in triplicate within five different experiments. Necrosis was assessed using the LDH activity assay as detailed in section 4.2.7. Each condition was performed in triplicate in three different experiments. For each assay, the results are expressed relative to controls (cells transfected with control siRNA or VO and treated with 0 nM T_3). The timeline of SGHPL-4 transfection, T_3 treatment and the subsequent experiments are summarised in **Figure 5-3**.
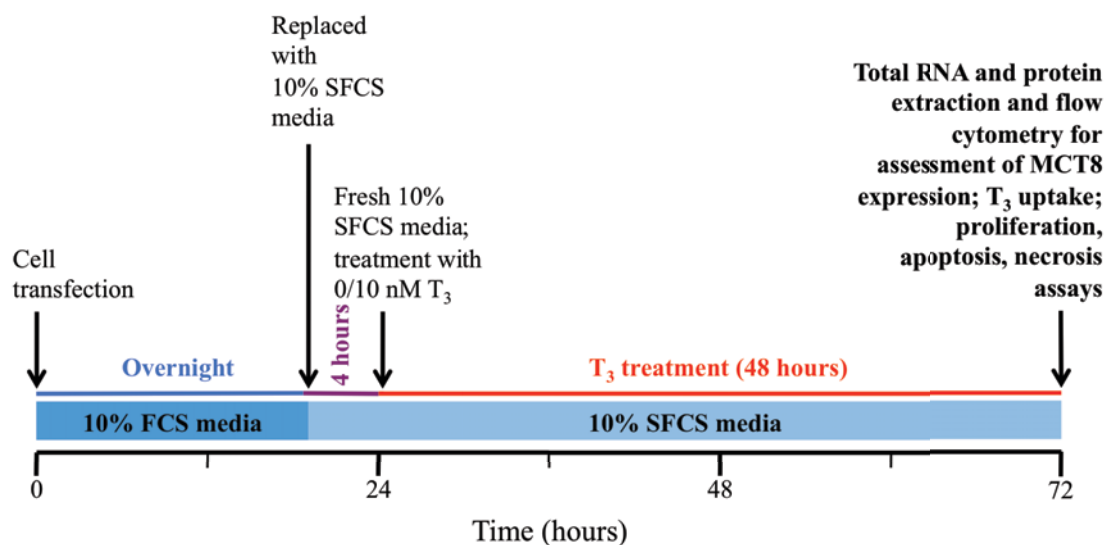
Figure 5-3: *Diagram of MCT8 silencing and upregulation experiments. SGHPL-4s were transfected with siRNA or plasmid DNA and incubated in 10% FCS-supplemented media in tissue culture plates overnight. The media was replaced with 10% SFCS-supplemented media. After a 4-hour washing period the media was replaced with fresh 10% SFCS media containing 0 or 10 nM T₃. Cell proliferation and apoptosis assays were performed 48 hours after T₃ treatment.*

5.2.4   Statistical analysis

Data were analysed using Minitab$^{®}$ statistical software (version 15). Residuals for all data sets passed the normality test as determined using the Kolmogorov-Smirnov test. When two groups were present, statistical significance was assessed by paired t test. When more than two groups were present, analysis of variance (ANOVA) was performed using the general linear model. Tukey all pairwise multiple comparisons post-hoc tests were used to assess differences between individual groups.

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5.3   Results

5.3.1   Effect of T$_3$ treatment on SGHPL-4s

As shown in **Table 5-2**, SGHPL-4s express TRα1, TRα2 and TRβ1, suggesting that they are sensitive to the actions of THs.

Table 5-2: *mRNA expression of TRs in SGHPL-4s compared with term placenta tissue*

Gene	Fold Change compared with term placenta
TRα1	0.185±0.054
TRα2	1.464±0.180
TRβ1	1.200±0.085

Values are means ±SEM

Initially the responsiveness of the EVT-like cell line, SGHPL-4, to treatment with different doses of T$_3$ in terms of invasive ability, proliferation and apoptosis was investigated. Furthermore, the expression of the TH responsive genes, ME1 and Cx43, and of the TH transporter MCT8 was assessed following T$_3$ treatment.

5.3.1.1   Effect of T$_3$ treatment on SGHPL-4 invasion

Treatment with 1 or 10 nM T$_3$ increased SGHPL-4 invasion by approximately 2-fold compared with treatment with 0 nM T$_3$ (**Figure 5-4A**). However, this trend was not statistically significant. A representative picture of SGHPL-4s that invaded through Matrigel® and adhered at the bottom of the tissue culture insert can be seen in **Figure 5-4B**.
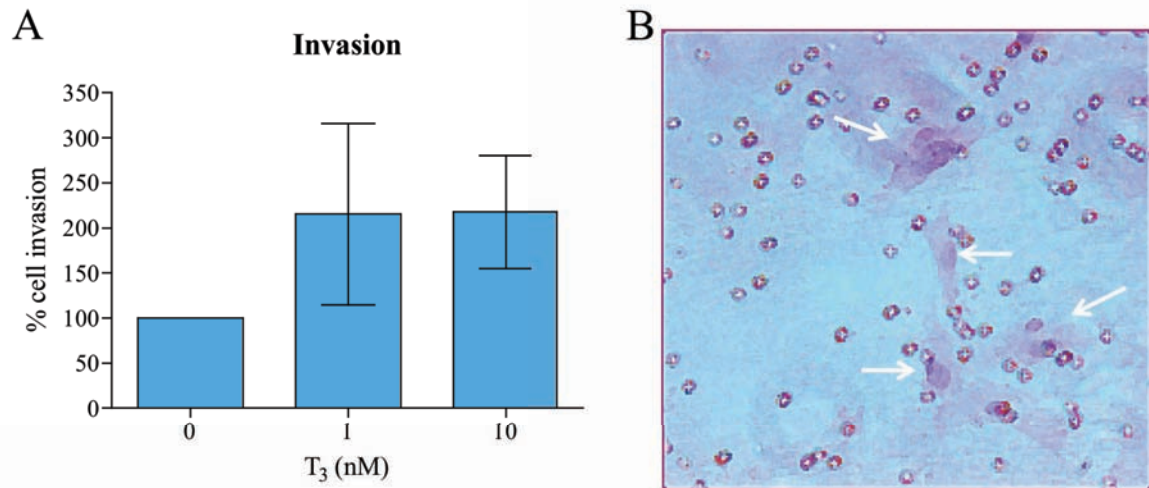
Figure 5-4: ***(A)*** *Effect of T_3 treatment on SGHPL-4 invasion through Matrigel[®]. Invasion was assessed 48 hours after treatment with T_3. The bars represent average of three experiments ±SEM. Within each experiment the result was normalised to the control group that was treated with 0 nM T_3.* ***(B)*** *SGHPL-4s that invaded through Matrigel[®] and adhered to the bottom of the tissue culture insert. Cells (indicated by white arrows) were fixed and stained with H&E. Photographed with a 20× objective lens.*

5.3.1.2   Effect of T_3 treatment on SGHPL-4 proliferation and apoptosis

T_3 treatment did not have an effect on either SGHPL-4 proliferation, assessed by the MTT assay, or on caspase 3/7 activity in SGHPL-4s (**Figure 5-5**).
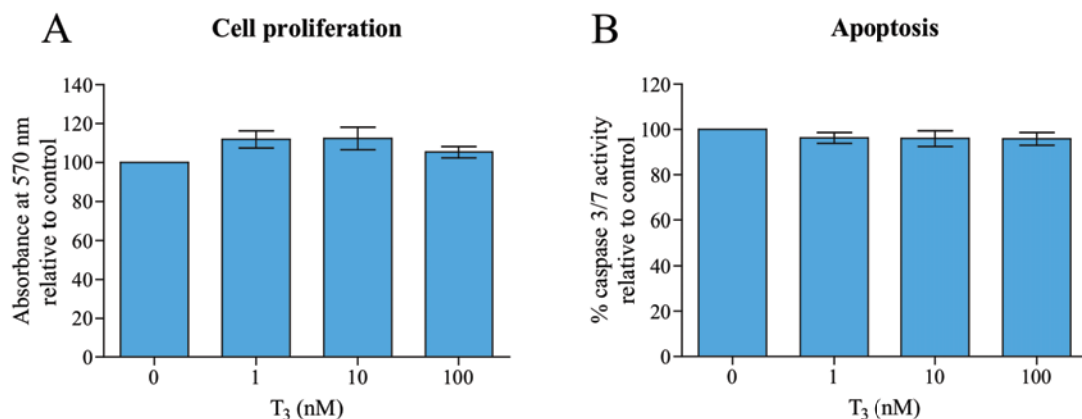
Figure 5-5: *Effect of T_3 treatment on SGHPL-4 proliferation* ***(A)*** *and apoptosis* ***(B)***. *SGHPL-4 proliferation and apoptosis were assessed 48 hours after treatment with T_3 using the MTT assay or the Caspase 3/7 activity assay, respectively. Within each experiment the result was normalised to the control group that was treated with 0 nM T_3. The bars represent average of four experiments ±SEM.*

5.3.1.3 Expression of TH responsive genes following T_3 treatment

The mRNA expression of the TH responsive genes ME1 and Cx43 was assessed following a time course of treatment with 0 or 10 nM T_3. Overall, T_3 treatment had no effect on the expression of either ME1 or Cx43, although the expression of both genes significantly changed with time ($P<0.05$ for both; **Figure 5-6**). Post-hoc analysis by Tukey tests revealed no significant differences between individual time points. These results suggest that the expression of ME1 and Cx43 in SGHPL-4s is not regulated by T_3.
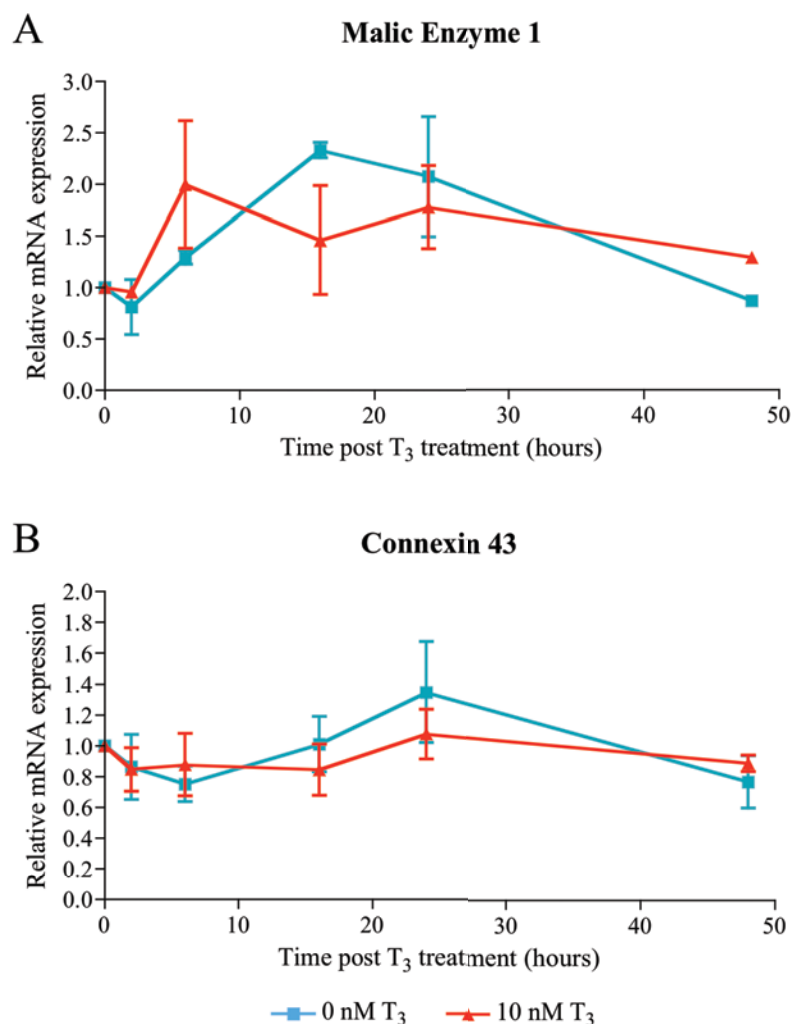
Figure 5-6: *Relative mRNA expression of the TH responsive genes Malic Enzyme 1 (ME1;* ***A)*** *and Connexin 43 (Cx43;* ***B)*** *in response to T_3 treatment. Relative expression of ME1 and Cx43 was assessed following a time course of treatment with 0 or 10 nM T_3. Expression was normalised to the housekeeping control 18S and was compared with the mean mRNA expression following 0 hours of treatment with T_3 that was given the arbitrary value of one. The points represent average of four experiments ±SEM.*

5.3.1.4   Expression of MCT8 following T_3 treatment

The mRNA expression of the TH transporter MCT8 was also assessed following a time course of treatment with 0 or 10 nM T_3 in order to find out if T_3 can regulate the expression of its transporter. Overall, MCT8 expression did not change with T_3 treatment but it was significantly altered with time (P<0.01; **Figure 5-7**). Post-hoc analysis by Tukey tests revealed no significant differences between individual time points.
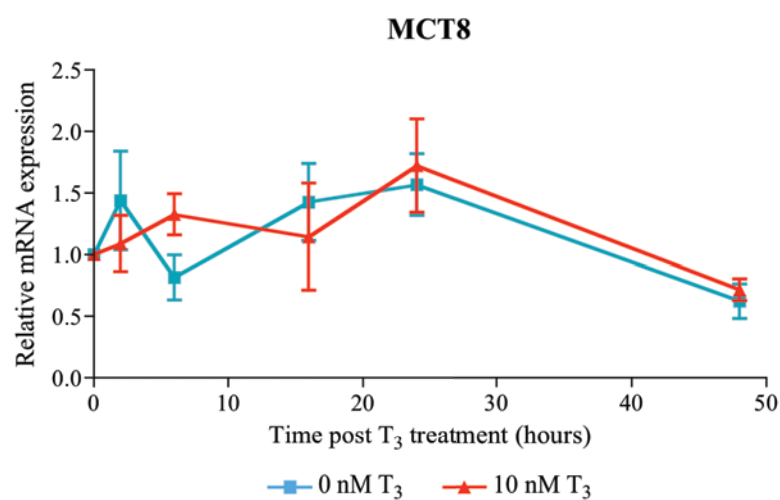
Figure 5-7: *Relative mRNA expression of MCT8 in response to T_3 treatment. Relative expression of MCT8 was assessed after a time course of treatment with 0 or 10 nM T_3. Expression was normalised to the housekeeping control 18S and was compared with the mean MCT8 expression following 0 hours of treatment with T_3 that was given the arbitrary value of one. The points represent average of four experiments ±SEM.*

5.3.2   Effect of silencing and upregulation of MCT8 expression in SGHPL-4s

5.3.2.1   Optimisation of MCT8 silencing and upregulation

In order to investigate the role of MCT8 in the EVT-like cell line, SGHPL-4, MCT8 expression was silenced or upregulated. In the first instance, transfection of SGHPL-4s with the Amaxa® electroporation method was optimised, followed by experiments to confirm changes in MCT8 expression following transfection.

5.3.2.1.1 Optimisation of transfection of SGHPL-4s

SGHPL-4s were transfected with pmaxGFP using a combination of two electroporation solutions and seven electroporation programs in order to identify the optimal conditions for transfection. The outcome of each condition on transfection efficiency and cell recovery is reported in **Table 5-3**. Following this experiment, programs X-001 and X-005 in combination with solution V were identified as the optimal conditions tested for the transfection of SGHPL-4s. Program X-001 and solution V were used in all subsequent experiments.

Table 5-3: *Cell recovery and transfection efficiency following electroporation.*

Solution	Program setting	% Cell recovery compared with control	% GFP positive cells
L	A-020	43	42
	T-020	18	59
	T-030	28	80
	X-001	36	57
	X-005	57	58
	L-029	52	49
	D-023	25	54
	No electroporation	66	0
	No DNA (T-020)	50	0
V	A-020	54	39
	T-020	25	87
	T-030	21	88
	X-001	**82**	**78**
	X-005	78	82
	L-029	53	69
	D-023	42	70
	No electroporation	83	0
	No DNA (T-020)	43	0
No electroporation; No DNA		100	0

5.3.2.1.2 Detection of endogenous MCT8 protein in SGHPL-4s

When SGHPL-4 cell lysates from cells transfected with MCT8 were probed with the 4790 antibody against MCT8, a strong band was detected at approximately 60 kDa

and more bands of higher molecular weight were seen between 150 and >250 kDa (**Figure 5-8A**). However, when cells transfected with VO, control siRNA or MCT8 siRNA were probed with the 4790 MCT8 antibody, there was no detectable band at the expected size even after a long exposure (60 minutes). Following repeated attempts to detect endogenous MCT8 in SGHPL-4 lysates an alternative antibody was used for the detection of endogenous MCT8 in SGHPL-4s (1306 Ab; **Figure 5-8B**). A band was detected at approximately 60 kDa in the sample that was transfected with MCT8, although weaker than the band observed when the 4790 antibody was used. Bands of higher molecular weight (between 150 and >250 kDa) were also present in that sample. A single band at approximately 60 kDa, representing endogenous MCT8 protein, was present in the samples transfected with VO or control siRNA. No bands were detected in the sample that was transfected with MCT8 siRNA, following a 60-minute exposure.
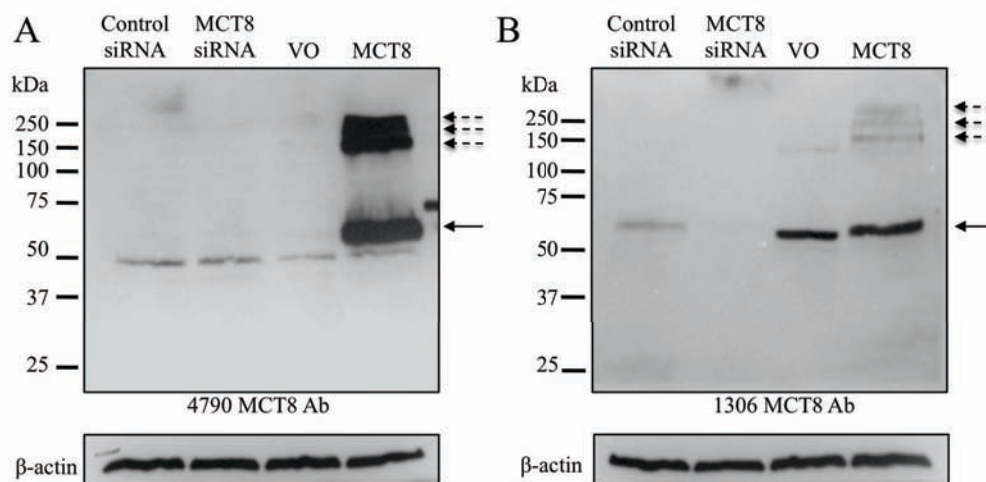
Figure 5-8: *Detection of endogenous MCT8 protein in SGHPL-4s by Western immunoblotting. Whole protein lysates of SGHPL-4s (70 µg) were harvested 48 hours after transfection with siRNA (control siRNA or MCT8 siRNA) or plasmid DNA (VO or MCT8) and they were probed with the 4790 MCT8 antibody (Ab)* ***(A)*** *or the 1306 MCT8 Ab* ***(B)****. Transfected MCT8 was detected at approximately 60 kDa (solid line arrows). More bands were present between approximately 150 and 250 kDa (dashed line arrows). Endogenous MCT8 was only detactable in SGHPL-4s using the 1306 MCT8 Ab as a single band running at the same size as transfected MCT8 (approximately 60 kDa). Immunoblotting for β-actin was used to assess protein loading.*

5.3.2.1.3   *Efficiency of upregulation of MCT8 expression*

The proportion of the cells expressing transfected MCT8 was assessed by flow cytometry (**Figure 5-9A**). Cells transfected with VO were used as negative control. Following transfection with HA-tagged MCT8, 41% of the cells were positively stained for HA. The increase in the expression of MCT8 protein in cells transfected with MCT8 compared with VO was confirmed by Western immunoblotting using the 4790 MCT8 antibody (**Figure 5-9B**). A band representing MCT8 was detected at 60 kDa and bands possibly representing MCT8 multimers were identified between 150 and >250 kDa in the sample transfected with MCT8. No band was present in the sample transfected with VO. In addition, transfected MCT8 protein was detected by immunofluorescent staining of the HA tag (**Figure 5-9C**).
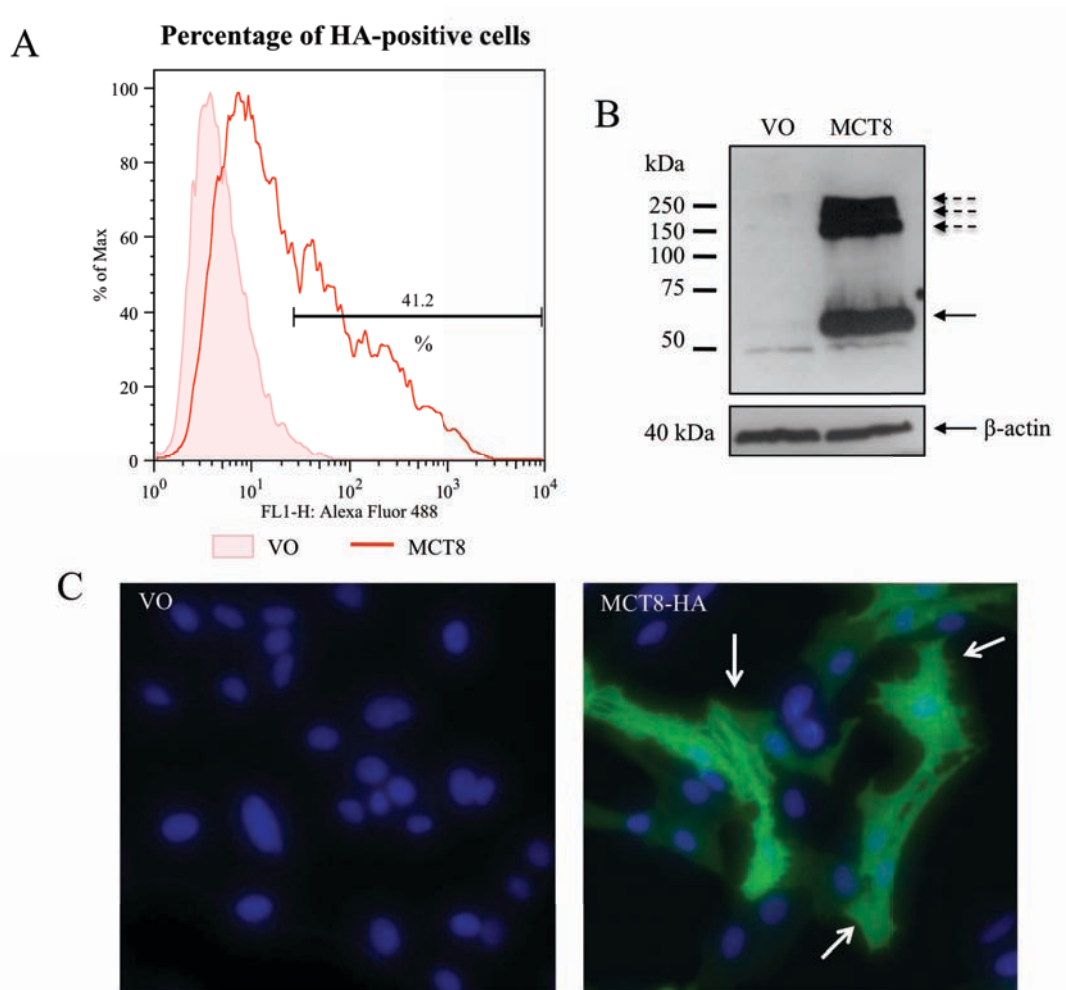
Figure 5-9: *Efficiency of MCT8 transfection. **(A)** The percentage of cells that expressed HA-tagged MCT8 was assessed by flow cytometry 72 hours post-transfection. The cells were probed with anti-HA antibody followed by Alexa Fluor 488 labelled secondary anti-mouse antibody. Cells transfected with VO were used as negative control. **(B)** Lysates of SGHPL-4s transfected with MCT8 or VO were probed with the 4790 MCT8 antibody followed by secondary anti-rabbit antibody conjugated with HRP. The band at 60 kDa representing the MCT8 monomer is indicated by solid line arrows. The bands representing MCT8 multimers are indicated by dashed line arrows. Immunoblotting for β-actin was used to assess protein loading. **(C)** Localisation of transfected MCT8 protein by immunofluorescence. SGHPL-4s transfected with MCT8-HA or VO were fixed 72 hours after transfection and were probed with antibody against the HA tag followed by secondary anti-rabbit antibody labelled with green-fluorescent Alexa Fluor 488 dye. Nuclei were stained blue using the Hoescht 33258 stain. The cells were photographed using a 40× objective lens. Cells expressing HA-tagged MCT8 are indicated by white arrows.*

5.3.2.1.4 Efficiency of silencing endogenous MCT8 expression

The silencing of MCT8 mRNA was assessed by TaqMan PCR 24 hours post-transfection. Endogenous MCT8 expression was reduced by approximately 70% in the samples transfected with MCT8 siRNA compared with those transfected with control siRNA ($P<0.05$; **Figure 5-10A**). MCT8 silencing at the protein level was confirmed 72 hours post-transfection by Western immunoblotting using the 1306 MCT8 antibody (**Figure 5-10B**). A band representing endogenous MCT8 was present at approximately 60 kDa in the sample that was transfected with control siRNA. There was no band detectable in the sample transfected with MCT8 siRNA, following exposure for 60 minutes.
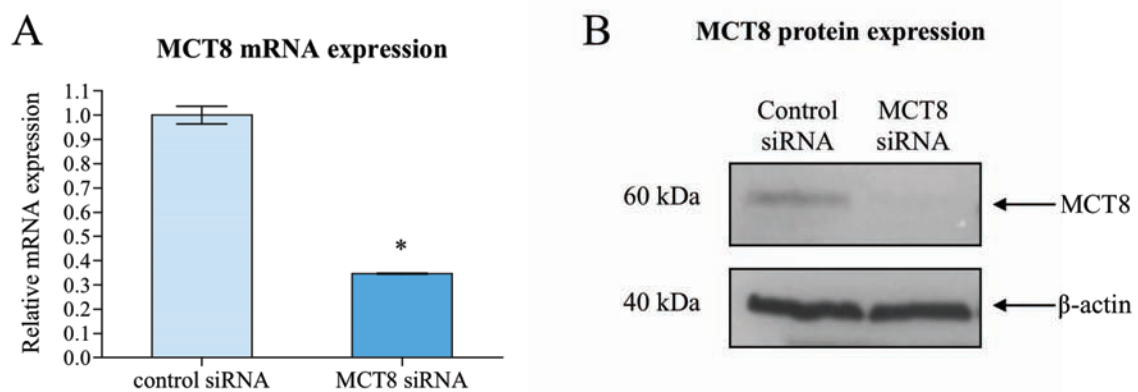
Figure 5-10: *Efficiency of MCT8 silencing. **(A)** Silencing of endogenous MCT8 mRNA expression was assessed by semi-quantitative PCR 24 hours after transfection. Bars represent average of two experiments ±SEM. Statistically significant differences are indicated by * (P<0.05). MCT8 expression was normalised to the housekeeping control 18S and was compared to the average expression in cells transfected with control siRNA that was given the arbitrary value of one. **(B)** Silencing of endogenous MCT8 protein was assessed by Western immunoblotting. Lysates of SGHPL-4s transfected with MCT8 siRNA or control siRNA were harvested 72 hours post-transfection and were probed with the 1306 MCT8 antibody followed by secondary anti-rabbit antibody conjugated to HRP. Immunoblotting for β-actin was used to assess protein loading.*

5.3.2.2 T_3 *uptake following changes in MCT8 expression*

The impact of MCT8 silencing or upregulation on T_3 uptake was assessed 72 hours post transfection. Overall, silencing of endogenous MCT8 significantly reduced T_3 uptake by SGHPL-4s (**Figure 5-11A**; $P<0.001$). When compared with control siRNA, transfection with MCT8 siRNA resulted in a 40% reduction in the proportion of $[^{125}I]$-T_3 that was retained intracellularly following 10 ($P<0.05$) or 30 minutes ($P<0.01$) of incubation with $[^{125}I]$-T_3. This effect remained significant following co-transfection of MCT8 siRNA with CRYM. Transfection with CRYM alone did not affect T_3 uptake when compared with cells transfected with control siRNA.

Upregulation of MCT8 expression resulted in an increase in T_3 uptake by SGHPL-4s (**Figure 5-11B**; $P<0.001$). Compared with transfection with VO, transfection with MCT8 resulted in a 25% increase in the proportion of $[^{125}I]$-T_3 that was retained intracellularly after a 5- or 10-minute incubation period ($P<0.05$ for both). However, this difference in T_3 uptake was not maintained following 30 minutes of incubation with $[^{125}I]$-T_3. Following co-transfection of MCT8 with CRYM, the increase in T_3 uptake was even more pronounced and it was sustained for up to 30 minutes, which was the longest incubation period tested. Transfection with CRYM alone did not affect T_3 uptake when compared with transfection with VO.
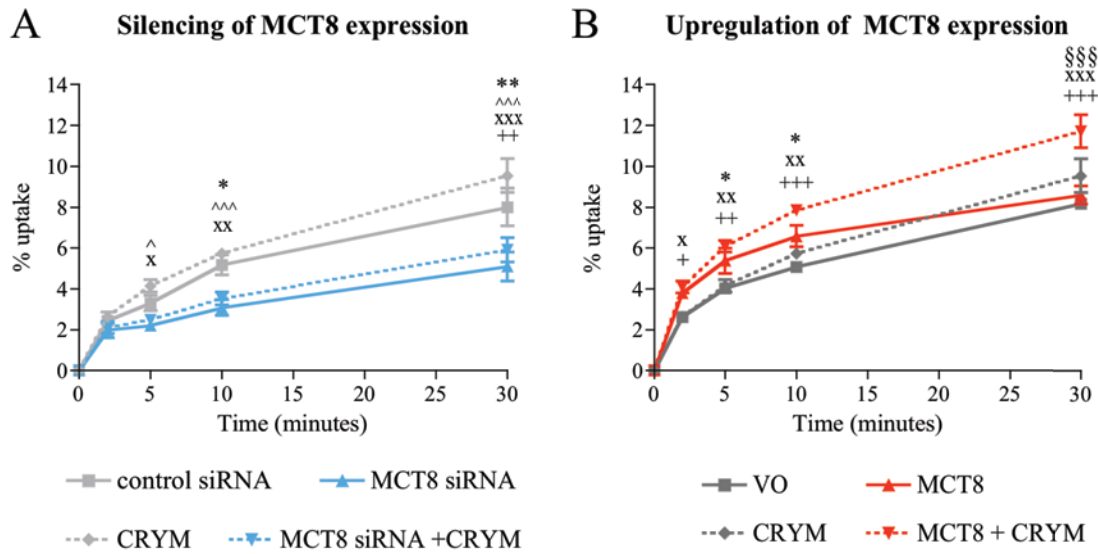
Figure 5-11: *T_3 uptake by SGHPL-4s 72 hours after MCT8 silencing **(A)** or MCT8 transfection **(B)**. SGHPL-4s were incubated for 0-30 minutes with $[^{125}I]$-T_3 and the amount of intracellular radioactivity was assessed. Points represent average of three experiments ±SEM. Statistically significant differences were assessed by ANOVA followed by Tukey posttests. **(A)** Statistically significant differences are indicated by * (control siRNA vs MCT8 siRNA), $^+$ (control siRNA vs MCT8 siRNA + CRYM), ^{x} (MCT8 siRNA + CRYM vs CRYM) and ^ (MCT8 siRNA vs CRYM). **(B)** Statistically significant differences are indicated by * (VO vs MCT8), $^+$ (VO vs MCT8 + CRYM), ^{x} (MCT8 + CRYM vs CRYM) and § (MCT8 vs MCT8 + CRYM). *P<0.05, **P<0.01 and ***P<0.001.*

5.3.2.3   MCT8 potentiates the pro-invasive effect of T_3 on SGHPL-4s

T_3 promotes invasion by primary EVTs (Oki *et al.*, 2004). As shown in section 5.3.1.1, there was a trend of increased invasion by SGHPL-4s following T_3 treatment, however this trend was not statistically significant. In order to investigate the hypothesis that MCT8 may amplify the effect of T_3 on invasion, invasion by SGHPL-4s was assessed following silencing or upregulation of MCT8 expression and treatment with 0 or 10 nM T_3. Silencing endogenous MCT8 expression did not significantly affect invasion by SGHPL-4s (**Figure 5-12A**). Following upregulation of MCT8 expression, treatment with 10 nM T_3 resulted in a 3.3-fold increase in invasion compared with treatment with 0 nM

T_3 (**Figure 5-12B**; $P<0.05$). In contrast, in cells that were transfected with VO, treatment with 10 nM T_3 resulted in a 1.8-fold increase in invasion compared with treatment with 0 nM T_3 (not statistically significant).
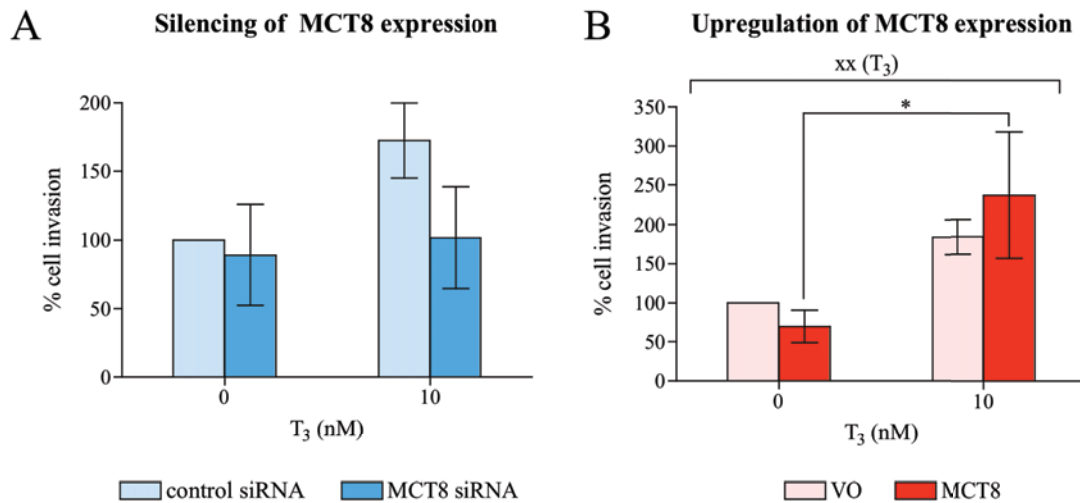
Figure 5-12: *SGHPL-4 invasion through Matrigel® following silencing of endogenous MCT8 expression **(A)** or transfection with MCT8 **(B)** and treatment with 0 or 10 nM T_3. Invasion was assessed 72 hours after transfection. Within each experiment, the number of invading cells was normalised to the average number of invading cells in the control groups that were transfected with control siRNA **(A)** or VO **(B)** and treated with 0 nM T_3. Bars represent average of five (silencing) or eight (upregulation) experiments ±SEM. Results following analysis by ANOVA are indicated by xx (P<0.01). Statistically significant differences between groups analysed by Tukey post-tests are indicated by *(P<0.05).*

5.3.2.4 MCT8 does not affect SGHPL-4 proliferation

As discussed in the previous chapter, upregulation of MCT8 resulted in decreased survival of primary CTs (section 4.3.3) and in reduced proliferation of the choriocarcinoma cell line JEG-3 (James *et al.*, 2009). In order to investigate whether MCT8 has a similar effect on SGHPL-4s, SGHPL-4 proliferation was assessed by MTT following silencing and upregulation of MCT8 expression and culture in the absence or

presence of T_3. In contrast with the results observed in other cell types, there was no significant difference in SGHPL-4 proliferation either with changes in MCT8 expression or with T_3 treatment (**Figure 5-13**).
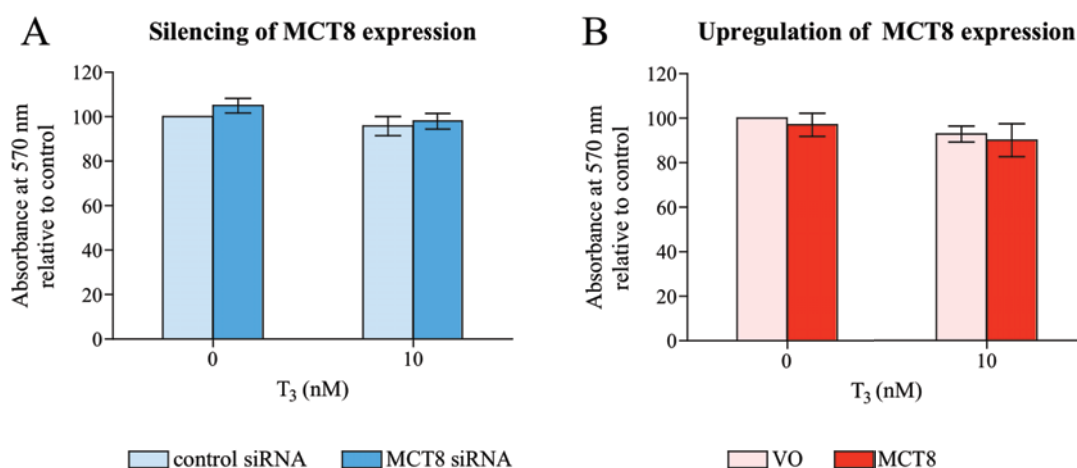
Figure 5-13: *SGHPL-4 proliferation following silencing of endogenous MCT8 expression (A) or transfection with MCT8 **(B)** and treatment with 0 or 10 nM T_3. Absorbance at 570 nm was determined 72 hours after transfection and was normalised to the absorbance in the control groups that were transfected with control siRNA **(A)** or VO **(B)** and treated with 0 nM T_3. Bars represent average of five experiments ±SEM.*

5.3.2.5   Silencing of MCT8 expression reduces SGHPL-4 apoptosis

The effect of MCT8 on SGHPL-4 survival was further assessed by investigating apoptosis in response to changes in the expression of MCT8 in the absence or presence of T_3 using the caspase 3/7 activity assay. Overall, silencing of MCT8 expression caused a reduction in caspase 3/7 activity (**Figure 5-14A**; $P<0.01$). Compared with control siRNA, transfection with MCT8 siRNA caused a 10% reduction in caspase 3/7 activity both in cells treated with 0 nM T_3 and in cells treated with 10 nM T_3 ($P<0.05$ for both). In contrast, upregulation of MCT8 expression did not have an effect on caspase 3/7 activity in SGHPL-4s (**Figure 5-14B**).
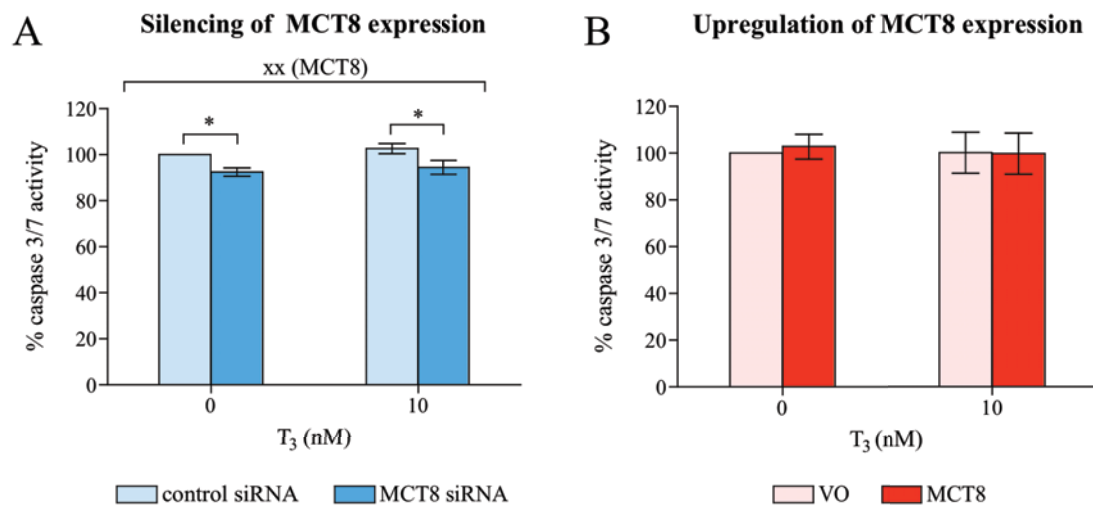
Figure 5-14: *SGHPL-4 apoptosis following silencing of endogenous MCT8 expression **(A)** or transfection with MCT8 **(B)** and treatment with 0 or 10 nM T_3. Apoptosis was assessed 72 hours after transfection by assessing the activity of caspase 3/7. Caspase 3/7 activity was normalised to the activity in the control groups that were transfected with control siRNA **(A)** or VO **(B)** and treated with 0 nM T_3. Bars represent average of five experiments ±SEM. ANOVA results are indicated by xx (P<0.01). Statistically significant differences between groups as assessed by Tukey post-tests are indicated by * (P<0.05).*

5.3.2.6   MCT8 does not affect necrosis of SGHPL-4s

In addition, the possibility that MCT8 causes necrosis of SGHPL-4s was investigated by assessment of the level of LDH activity in cell culture media following MCT8 silencing or upregulation. There was no change in cell necrosis either with changes in MCT8 expression or with T_3 treatment (**Figure 5-15**).
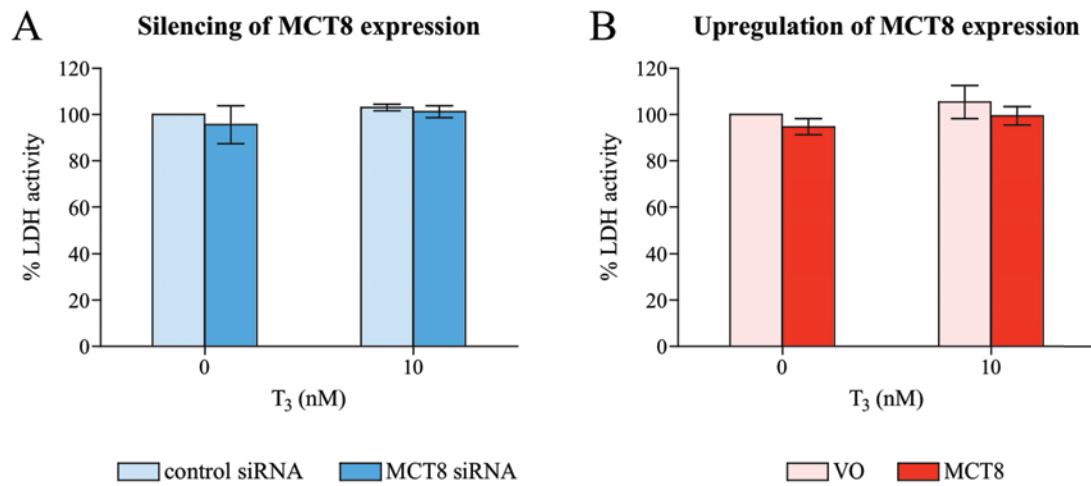
Figure 5-15: *SGHPL-4 necrosis following silencing of endogenous MCT8 expression **(A)** or transfection with MCT8 **(B)** and treatment with 0 or 10 nM T₃. Necrosis was assessed 72 hours after transfection by determining the LDH) activity in the cell culture media. LDH activity was normalised to the activity in the control groups that were transfected with control siRNA **(A)** or VO **(B)** and treated with 0 nM T₃. Bars represent average of three experiments ±SEM.*

5.4   Discussion and conclusions

In this study, the role of MCT8 in EVTs was investigated using the EVT-like cell line, SGHPL-4. According to the findings presented here, MCT8 mediates the pro-invasive effect of T_3 on SGHPL-4s. Furthermore, silencing MCT8 expression reduces SGHPL-4 apoptosis in a T_3-independent manner.

5.4.1   Use of SGHPL-4s as an EVT-like cell line model

The development of EVT-like cell lines has been useful for the investigation of EVT cell biology and function, particularly because of the difficulties in obtaining first trimester placental tissue for primary cultures. However, there is ongoing debate about the validity of the use of cell lines as models of EVTs (Shiverick *et al.*, 2001). SGHPL-4s, an immortalised cell line derived from primary EVTs, express MHC Class I antigens, human placental lactogen, human chorionic gonadotropin, integrin $\alpha 1$ and cytokeratin 7, which are also expressed in EVTs and they therefore have characteristics of EVTs (Choy *et al.*, 2000, Lash *et al.*, 2007). However, in contrast to EVTs, SGHPL-4s do not express HLA-G (Lash *et al.*, 2007). Furthermore, the responsiveness of cell lines to stimuli such as hypoxia and TGF-β1 has been shown to differ from primary EVT cultures (Lash *et al.*, 2007).

5.4.2   Effects of T_3 treatment on SGHPL-4 cells

Prior to investigating the role of MCT8 in SGHPL-4s, the effects of T_3 treatment were assessed. The presence of mRNA encoding TRα1, TRα2 and TRβ1 in SGHPL-4s suggests that these cells can respond to T_3 treatment. Treatment with T_3 resulted in increasing trend in cell invasion by SGHPL-4s, which did not reach statistical

significance, whilst SGHPL-4 proliferation and apoptosis were not affected by T_3 treatment. In a previous report from our group, T_3 increased the motility of SGHPL-4 cells but did not affect significantly the number or length of the invasive processes formed (Barber *et al.*, 2005). Furthermore, in the report by Barber *et al.* T_3 treatment had an anti-proliferative effect on SGHPL-4s. The discrepancy on the effect of T_3 on SGHPL-4 proliferation may be due to the differences in the cell culture conditions and in the time point that was used to assess the effect of T_3 on cell proliferation. In the study by Barber *et al.* the cells were cultured in 0.5% FCS, and were therefore exposed to lower concentrations of all serum constituents, in contrast to 10% SFCS, which was used in the experiments described here and is only devoid of lipophilic substances, such as hormones. Furthermore, in the study by Barber *et al.* effects were assessed 72 hours after treatment with T_3 instead of 48 hours that was the time point used in the experiments described in this thesis (Barber *et al.*, 2005). Previous studies have shown that T_3 promotes invasion (Oki *et al.*, 2004) and decreases apoptosis (Laoag-Fernandez *et al.*, 2004) in primary EVTs. In contrast, T_3 had a modest effect on SGHPL-4 invasion, which did not reach statistical significance. This together with the differential effect of T_3 on apoptosis of EVTs and SGHPL-4s suggests that there may be differences between the T_3 responsiveness of EVTs and SGHPL-4s. It has to be noted, however, that in the quoted studies, EVTs were cultured in media supplemented with 4% FCS, which would therefore contain more hormones compared with media supplemented with 10% SFCS.

The expression of the TH responsive genes Cx43 and ME1 was investigated following treatment with T_3 in order to demonstrate the induction of genomic TH actions by T_3 treatment in SGHPL-4s. It has been shown that TRs bind to a response element in the promoter of the rat gap junction protein, Cx43, (Stock and Sies, 2000) suggesting that

THs can regulate the expression of Cx43. This was confirmed by further evidence that treatment with T_3 increased the expression of Cx43 in rat liver epithelial cells (Stock *et al.*, 1998), in cultured rat heart myocytes (Tribulova *et al.*, 2004) and in mouse Sertolli cells (Gilleron *et al.*, 2006). In addition, TH response elements have been identified in the promoter region of the human cytosolic enzyme ME1 (Gonzalez-Manchon *et al.*, 1997). However, the effect of genomic TH action on ME1 expression is cell type-specific with T_3 stimulating an increase in ME1 expression in chick embryo hepatocytes whereas it had little or no effect on ME1 expression in chick embryo fibroblasts and in quail QT6 cells (Hillgartner *et al.*, 1992). Data on the regulation of ME1 expression by THs suggest that cell-type specific differences may be mediated by non-receptor proteins that augment the transcriptional activity of TRs in hepatocytes (Hillgartner *et al.*, 1992). In SGHPL-4s, treatment with T_3 did not affect the mRNA expression of either Cx43 or ME1, which is consistent with previous findings in primary cultures of human term CTs (Barber, 2005). Therefore, it was not possible to demonstrate genomic action of TH in SGHPL-4s using these genes.

5.4.3   Changes in SGHPL-4 MCT8 expression and impact on T_3 uptake

In this study, MCT8 expression was silenced or upregulated in order to elucidate the role of MCT8 in SGHPL-4s. The validation of MCT8 silencing at the protein level was hindered by the problems in detecting endogenous MCT8 expression in SGHPL-4s using the 4790 MCT8 antibody produced by our group (Loubière *et al.*, 2010), even though this antibody has been successfully used to detect endogenous MCT8 protein in other cell types, such as CTs (see Chapters 3 and 4) and N-Tera-2 cells. However, TaqMan PCR revealed that MCT8 mRNA is present at high levels in SGHPL-4s, and furthermore there was a reduction of MCT8 mRNA following transfection with MCT8.

Despite the problems in the detection of endogenous MCT8 protein in SGHPL-4s using the 4790 antibody, detection was possible using the 1306 MCT8 antibody and the downregulation of MCT8 following transfection with MCT8 siRNA was confirmed at the protein level. It is not clear why there is a difference in the detection of MCT8 using the two different antibodies. It is possible that the difference is associated with the different epitopes targeted by the two antibodies. Antibody 4790 recognises an epitope at the N-terminus of the MCT8 protein, whilst antibody 1306 recognises an epitope on the C-terminus of MCT8.

The functional impact of changes in MCT8 expression was confirmed by changes in T_3 uptake by SGHPL-4s. Furthermore, co-transfection with CRYM was used to inhibit T_3 efflux. CRYM is a cytosolic protein that binds to T_3 (Vie *et al.*, 1997) and increases the concentration of intracellular T_3 (Suzuki *et al.*, 2007). It is thought that CRYM may regulate TH action by regulating they cytoplasmic and nuclear distribution of T_3 and thus regulating the expression of T_3-responsive genes (Suzuki *et al.*, 2007). In humans, CRYM mRNA has been detected in skeletal muscle, heart, kidney, brain, retina and in the inner ear (Kim *et al.*, 1992) and CRYM mutations have been associated with deafness (Abe *et al.*, 2003, Oshima *et al.*, 2006). Silencing of MCT8 expression considerably reduced T_3 uptake by SGHPL-4s and this difference was maintained following incubation with $[^{125}I]$-T_3 for 30 minutes. This suggests that T_3 uptake by SGHPL-4s transfected with MCT8 siRNA does not reach the same level of uptake as in cells with basal MCT8 levels, at least in the timeframe that was investigated. The impact of MCT8 silencing on T_3 uptake is highlighted by the inability of cells transfected with MCT8 siRNA to accumulate higher levels of intracellular T_3 following co-transfection with CRYM. The capacity of SGHPL-4s for T_3 uptake following MCT8 silencing amounted to 60% of the uptake by cells

transfected with control siRNA. Since, it was shown that MCT8 silencing in SGHPL-4s resulted in undetectable MCT8 protein, other TH transporters present in SGHPL-4s are probably responsible for the residual T_3 uptake following MCT8 silencing.

Transfection of SGHPL-4s with human wild-type MCT8 resulted in an initial increase in T_3 uptake by SGHPL-4s, which however was not maintained following a longer (30 minute) incubation with $[^{125}I]$-T_3. Although an increase in MCT8 expression results in increased ability of the cells to uptake T_3, it does not affect the amount of T_3 that was retained intracellularly when transport was allowed to occur for longer and equilibrium was approached due to T_3 efflux taking place. This was confirmed when T_3 uptake by SGHPL-4s that were co-transfected with MCT8 and CRYM was investigated. In these cells, the proportion of T_3 that was retained intracellularly following 30 minutes of incubation with $[^{125}I]$-T_3 was higher compared with cells transfected with MCT8. This result confirms that increased T_3 efflux limits the amount of T_3 that is retained in cells transfected with MCT8 following long incubations with $[^{125}I]$-T_3. However, when T_3 efflux in cells transfected with MCT8 is prevented by co-transfection with CRYM, intracellular accumulation of $[^{125}I]$-T_3 continues to increase after a 30-minute incubation period and equilibrium is not reached.

5.4.4   MCT8 potentiates the pro-invasive effect of T_3 on SGHPL-4s

As discussed in section 5.4.1, treatment with T_3 results in an increasing trend in SGHPL-4 invasion through Matrigel$^®$. Transfection with MCT8 amplifies the pro-invasive effect of T_3 on SGHPL-4s. Conversely, silencing of MCT8 expression attenuates the pro-invasive effect of T_3. These results suggest that MCT8 may play a role in regulating invasion into the maternal endometrium by controlling T_3 transport into EVTs.

Invasion of EVTs in the maternal endometrium during early pregnancy is critical for normal placental development. Deficient EVT invasion has been associated with malplacentation and pregnancy complications such as IUGR (Brosens *et al.*, 1977), pre-eclampsia (Brosens *et al.*, 1972), preterm delivery (Norwitz, 2006) and late ($\geq$13 weeks) but not early (<12 weeks) pregnancy loss (Ball *et al.*, 2006a, Ball *et al.*, 2006b). Maternal hyperthyroidism has been associated with IUGR and pre-eclampsia (Mestman, 2004), whilst maternal subclinical hypothyroidism has been associated with increased risks of miscarriage, placental abruption and preterm delivery (Abalovich *et al.*, 2002, Casey *et al.*, 2005). These observations suggest that an optimal supply of maternal THs is important for normal placental development.

In accordance to previous reports (Barber *et al.*, 2005, Oki *et al.*, 2004), the results presented in this chapter suggest that THs may affect early placental development by promoting invasion by EVTs. Furthermore, they suggest that changes in the expression of the TH transporter MCT8 can regulate the effect of T_3 on EVT invasion by controlling intracellular T_3 availability. The expression of MCT8 and of other TH transporters that have been localised in EVTs, such as MCT10 and OATP1A2 (Chapter 2), may thus be important in the regulation of EVT invasion in early pregnancy. Further studies would be necessary to further elucidate the mechanisms that regulate the pro-invasive effect of T_3 on EVT invasion and the importance of MCT8 in mediating the action of T_3. Assessment of the motility of the cells by time-lapse microscopy can be used to support the evidence that MCT8 potentiates the pro-invasive effect of T_3 on SGHPL-4s. Deiodinase enzymes may play a role in the regulation of the pro-invasive effect of T_3. A previous report suggests that T_3 treatment does not alter the mRNA expression levels of D2 and D3 in primary cultures of term CTs (Chan *et al.*, 2003). Assessment of the expression and

activity of deiodinase enzymes following changes in MCT8 expression and treatment with T$_3$ would help elucidate the possibility that there is lack of regulation by deiodinases following increased T$_3$ uptake by SGHPL-4s.

T$_3$ has also been shown to have a pro-invasive effect on the human hepatocellular carcinoma cell line, HepG2 (Chen *et al.*, 2008b). In the report by Chen *et al.*, it was shown that T$_3$ promotes invasion by upregulating the expression of furin, a protease that activates matrix metalloproteinases (MMP) 2 and 9. Furin is present in the human placenta and its expression is higher in the first trimester (Zhou *et al.*, 2009). Using the EVT-like cell line HTR-8, Zhou *et al.* have shown that furin upregulates HTR8 invasion through Matrigel$^®$ via upregulation of MMP-9 activity. It is therefore possible that T$_3$ exerts its pro-invasive effect on EVTs via upregulation of furin expression, which in turn may upregulate the activity of MMP-9 resulting in increased invasion.

5.4.5   Effects of MCT8 on SGHPL-4 growth

MCT8 did not affect SGHPL-4 proliferation, as assessed by MTT assay. However, silencing of MCT8 expression decreased apoptosis independently of T$_3$ treatment, assessed by changes in caspase 3/7 activity, in contrast with upregulation of MCT8 expression, which did not affect apoptosis. In contrast to the effect of MCT8 on CT survival (Chapter 4) and on the proliferation of JEG-3 and N-Tera-2 cells (James *et al.*, 2009), this is the only cell type where we did not observe an effect of MCT8 on cell number. Furthermore, this is the first time that MCT8 is reported to affect apoptosis. At present, the mechanisms that are involved in the T$_3$-independent effects of MCT8 are unknown. It is therefore difficult to postulate on the molecular pathways that may be responsible for the differential effect of MCT8 on SGHPL-4s compared with the other

cell types that have been investigated so far. However, it has to be noted that in contrast to JEG-3, which is a proliferating choriocarcinoma cell line, SGHPL-4 cells have an invasive phenotype and a lower proliferating rate. Therefore, the molecular apparatus that controls cell proliferation might be different in SGHPL-4s compared with JEG-3 cells. In future work, assessment of the effects of MCT8 on cell cycle in different cell types could help elucidate the effects of MCT8 on cell survival and proliferation and could lead to the identification of the pathways that are regulated by MCT8. Furthermore, it would be important to confirm these findings using primary cultures of EVTs, especially since that there was no effect of T_3 on SGHPL-4 apoptosis is contrast to what has been previously reported using primary EVT cultures (Laoag-Fernandez *et al.*, 2004).

5.4.6   Conclusions

The data presented in this chapter demonstrate that changes in the expression of MCT8 affect the EVT-like cells, SGHPL-4s, via both T_3-dependent and T_3-independent mechanisms. These observations are consistent with the hypothesis that MCT8 may mediate the promoting effect of TH on the invasive ability of EVTs and EVT-like cells. Furthermore, the T_3-independent effect of MCT8 silencing on SGHPL-4 apoptosis is consistent with the postulation that MCT8 may transport other compounds in addition to THs. The role of THs and of MCT8 on the regulation of EVT apoptosis and invasiveness needs to be investigated further in order to confirm these findings on primary cultures of EVTs and to elucidate the mechanisms that mediate these effects. Further understanding of these mechanisms will help to better define the contribution of MCT8 and THs in the regulation of EVT invasion into the maternal myometrium and transformation of the maternal spiral arteries, events that are critical for normal placentation.

Chapter 6: The Role of MCT8 in Placental Development Using

a MCT8-knockout Mouse Model

6.1   Introduction

In the previous chapters, the effect of MCT8 on placental development was investigated using *in vitro* approaches. The outcomes of these experiments were that MCT8 affects both EVT-like cells and CT cells via either T_3-dependent or T_3-independent mechanisms. MCT8 promotes the pro-invasive effect of T_3 on invasion by EVT-like cells whilst MCT8 knockdown decreases apoptosis of EVT-like cells independently of T_3. Furthermore, MCT8 appears to impair the survival of CT cells regardless of T_3 availability. These results suggest that MCT8 may play a role in placentation by regulating trophoblast invasion and placental growth and this could be further investigated by assessing the effect of MCT8 on placental development *in vivo*.

Recently, two MCT8-deficient mice models have been developed by two different laboratories (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007). Both the models have revealed that knockout of MCT8 in mice results in changes in TH metabolism similar to the ones observed in patients affected by MCT8 mutations. Serum T_4 is reduced whilst serum T_3 is increased in MCT8-null mice compared with wild-type littermates. However, unlike the situation in human patients affected by MCT8 mutations, who suffer from severe neurological impairment and locomotion deficits, MCT8-deficient mice do not exhibit any overt neurodevelopmental disorders. Furthermore, there are no differences in the fertility and reproduction of MCT8-deficient mice and no changes in growth rate postnatally. However, the effect of MCT8 deficiency on fetoplacental growth has not been investigated so far.

Mouse models are often employed to study pathways that may affect fetoplacental development (Watson and Cross, 2005). There are both similarities and differences

between human and mouse placental structures (**Figure 6-1**). The labyrinth region of the mouse placenta is thought to be analogous to the chorionic villi in humans (Rossant and Cross, 2001). In both cases, a ST cell layer is in direct contact with maternal blood and controls transplacental transport. The middle region of the mouse placenta, the junctional zone, consists of spongiotrophoblast cells. Cells from the junctional zone are thought to differentiate to polyploid trophoblast giant cells that are found in the decidua basalis, the outer region of the mouse placenta (Rossant and Cross, 2001). Trophoblast giant cells mediate the invasion of the maternal uterus. Therefore, the junctional zone of the mouse placenta is thought to be analogous to the CT columns that are found in the chorionic villi of the human placenta and the trophoblast giant cells are thought to have similar behaviour and function in the mouse placenta as EVTs in human (Rossant and Cross, 2001).
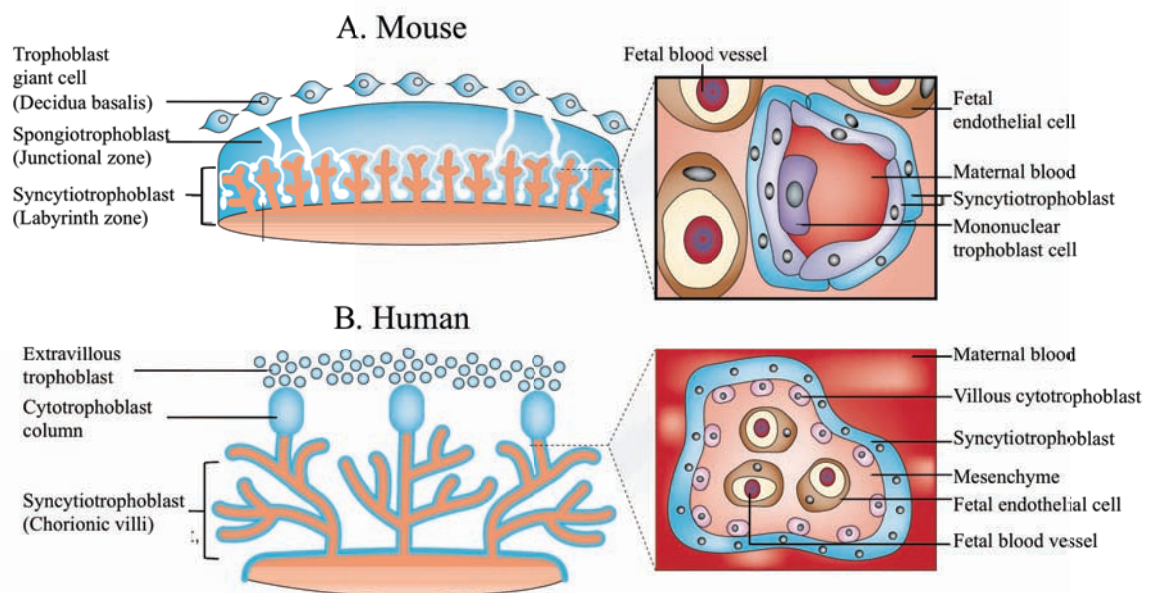
Figure 6-1: *Comparative diagram of the structure of mouse **(A)** and human **(B)** placenta. The main cell types found in each region are annotated in the left panel. The right panel shows a cross-section of the fetal-maternal interface. Figure adapted from (Rossant and Cross, 2001).*

In the study presented in this chapter the MCT8 knockout mouse model produced by Dr H Heuer's lab (Trajkovic *et al.*, 2007) was used to investigate the effect of MCT8 deficiency on fetoplacental development. For the generation of MCT8-deficient mice, a 33 bp fragment of exon 2, corresponding to nucleotides 758-790 of the MCT8 cDNA clone, was replaced by an IRES-LacZ Neo Insert containing a polyadenylation signal at the 3' end (**Figure 6-2**) (Trajkovic *et al.*, 2007). The effects of MCT8 were assessed at gestational day 14.5 (E14.5), which is before the onset of fetal TH production in mice (De Felice *et al.*, 2004), and at gestational day 18.5 (E18.5), which is after the onset of TH production and one day before delivery in mice. Fetal and placental weights and the morphology of the placenta were assessed in male MCT8 knockout and male wild-type fetuses in order to investigate the hypothesis that MCT8 influences placental development.
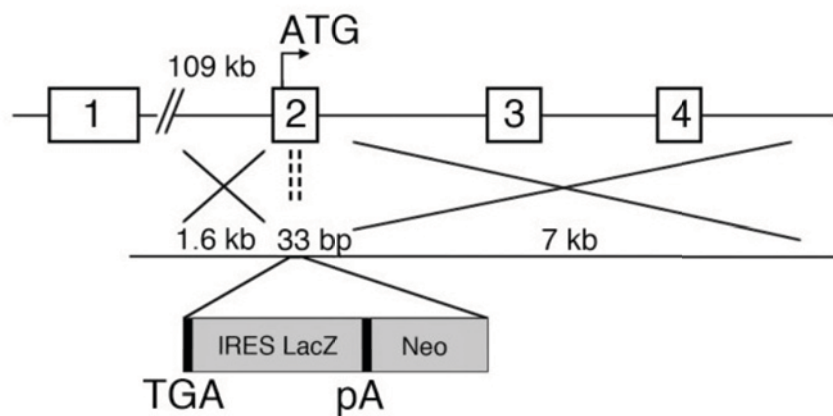
Figure 6-2: *Method used for the generation of MCT8 knockout mice. A fragment of exon 3 (33 bp) was replaced by an IRES-LacZ Neo insert. ATG: start codon; TGA: stop codon; pA: polyadenylation signal. Figure from (Trajkovic et al., 2007).*

6.2   *Materials and Methods*

Animal matings, euthanisation, dissection and genotyping were carried out during a visit to Dr H Heuer's laboratory in the Leibniz Institute for Age Research/Fritz Lipmann Institute, Gena, Germany with approval by the Animal Welfare Committee of the Medizinische Hochschule Hannover, Germany. Further experiments were performed in the Institute of Biomedical Research, Birmingham, UK.

6.2.1   Matings

Heterozygous female carrier mice (MCT8$^{+/-}$; n=6) were mated with male knockout mice (MCT8$^{-/0}$) in order to generate male knockout (MCT8$^{-/0}$), male wild-type (MCT8$^{+/0}$), heterozygous female (MCT8$^{+/-}$) and female knockout (MCT8$^{-/-}$) offspring at ratios of 1:1:1:1. Changes in fetoplacental weights and stereology of the placenta were compared between MCT8$^{-/0}$ and MCT8$^{+/0}$ fetuses.

6.2.2   Euthanisation and dissection

Two of the pregnant dams were euthanised at E14.5 and four were euthanised at E18.5. All pregnant dams were euthanised by CO_2 administration and they were weighed. The abdominal cavity was dissected and the uterine horns were retrieved. Each fetus and placenta was dissected out separately in a Petri dish containing PBS. Fetuses were weighed and the tails were biopsied for genotyping in order to determine the sex and the MCT8 status of each fetus. Each placenta was cleared of any uterine muscle and yolk sac and was weighed. Half of the placenta was fixed overnight at 4°C in paraformaldehyde/PIPES fixative (pH 7.4) containing 4% (w/v) paraformaldehyde, 3%

(w/v) PIPES, 3% (w/v) PVP40 and 0.02% (w/v) $CaCl_2$ for histological assessment. The other half was immediately frozen for RNA extraction.

6.2.3   Genotyping

6.2.3.1   DNA extraction

DNA was extracted from the tails in order to determine the sex and MCT8 status of the fetuses by PCR. The tails were digested by incubating overnight at 55°C, with shaking at 1,000 rpm, in buffer (0.71 ml per sample) containing 50 mM Tris/HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.4% SDS and Proteinase K (0.53 mg/ml; Roth, Germany). Following digestion, 0.25 ml of 6 M NaCl were added to each sample and the samples were shaken (1,000 rpm) for 5 minutes at 55°C. The samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant, containing the DNA, was transferred to new tubes. Isopropanol (0.6 ml) was added to each sample, in order to precipitate the DNA, the solutions were mixed by inverting and were centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 1 ml 70% (v/v) ethanol by centrifugation at 13,000 rpm for 10 minutes. The pellet was air-dried and resuspended in 100 μl Tris/HCl (10 mM; pH 8.0). The samples were shaken (1,000 rpm) at 55°C for 2 hours and diluted 1:30 with molecular quality water.

6.2.3.2   Sex determination

The sex of each fetus was determined by PCR amplification of a 433 bp-long sequence in the sex-determining region of chromosome Y (SRY) using the primers shown in **Table 6-1**. A PCR product would only be present if the DNA originated from a male fetus, while absence of a product indicated that the DNA belonged to a female fetus. DNA

was amplified using the *Taq* PCR Core Kit (Qiagen). DNA (3 μl) was mixed with 3

pmoles of each primer, dNTPs (2 nmoles of each), Buffer (1 μl), Q solution (2 μl) and

Taq polymerase (1 unit) in a total volume of 10 μl. The samples were heated at 94°C for 3

minutes followed by 35 cycles at 94°C for 90 seconds, 59°C for 60 seconds and 72°C for

60 seconds. They were incubated at 72°C for 7 minutes before cooling at 10°C.

Table 6-1: *Primers for SRY genotyping*

Forward Primer	Reverse Primer	Amplicon length (bp)
TGGTGAGAGGCACAAGTTGG	CTGCTGGTGGTGGTTATGGA	433

6.2.3.3 MCT8 genotyping

Three primers were used to detect the targeted allele (containing the IRES-LacZ

Neo Insert) and the wild-type allele (**Table 6-2**): two separate forward primers,

recognising the wild-type allele or the targeted allele, and a common reverse primer that

recognised both the wild-type and the targeted allele. The expected size of the PCR

products was 450 bp for the wild-type allele and 680 bp for the targeted allele. DNA (2

μl) was mixed with 5 pmoles of each primer, dNTPs (1 nmole of each), TEMPase buffer

with 15 mM MgCl2 (1 μl) and 0.5 units of TEMPase polymerase (all from Ampliqon,

Denmark) in a total volume of 10 μl. The samples were heated at 95°C for 15 minutes

followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30

seconds. They were incubated at 72°C for 5 minutes before cooling at 10°C. The SRY and

MCT8 PCR products were electrophoresed through a 1% agarose gel and were visualised

using a UV transilluminator. Representative gels for SRY and MCT8 genotyping are

shown in **Figure 6-3**. The genotyping results for all litters are shown in **Table 6-3**.

Table 6-2: *Primers for MCT8 genotyping*

Allele	Forward Primer	Reverse Primer	Amplicon length (bp)
Wild-type	TGTGAGTATATTCACTGACC GTTTG	CAATTCAATGGTCAAAGCA GGACTG	450
Targeted	GGGCCAGCTCATTCCTCCCA CTCAT	CAATTCAATGGTCAAAGCA GGACTG	680

Table 6-3: *Genotyping of fetuses harvested at gestational days E14.5 and E18.5*

Dam ID	Total N of fetuses	N of MCT8$^{+/-}$ fetuses	N of MCT8$^{-/-}$ fetuses	N of MCT8$^{+/0}$ fetuses	N of MCT8$^{-/0}$ fetuses
E14.5					
769	12	1	2	1	8
768	12	7	2	2	1
E18.5					
756	11	3	4	4	0
657	13	4	2	7	0
679	9	2	1	2	4
749	14	4	4	3	3
Total	71	21 (29.6%)	15 (21.1%)	19 (26.8%)	16 (22.5%)
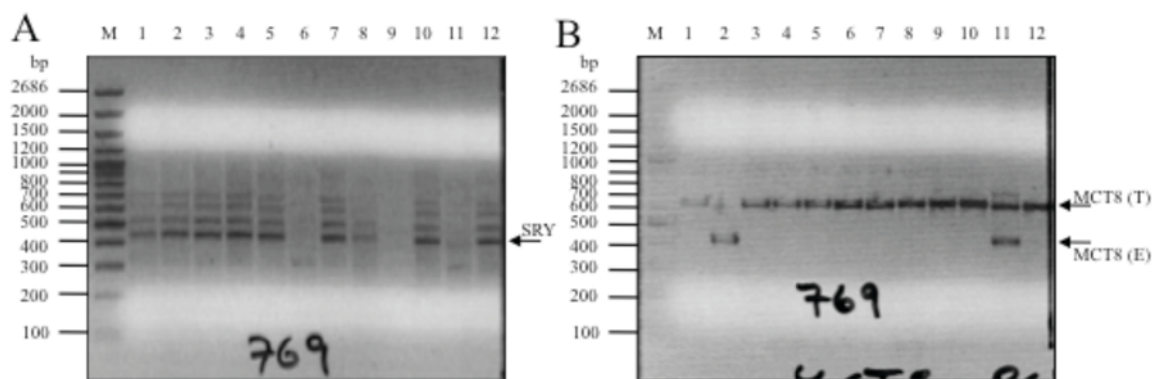
Figure 6-3: *Genotyping results for litter 769. **(A)** SRY genotyping: The presence of a 433 bp long species indicated the presence of the Y chromosome. Samples in lanes 1, 2, 3 ,4 , 5, 7, 8, 10 and 12 were identified as originating from male fetuses. Samples in lanes 6, 9 and 11 were from females fetuses. **(B)** MCT8 genotyping: The presence of a band at 450 bp indicated that the endogenous wild-type allele (E) was present, whilst the presence of a band at 680 bp indicated that the the target knockout allele (T) containing the lacZ/neomycin reporter casette was present. Samples in lanes 1, 3-10 and 12 were from MCT8 knockout fetuses. The sample in lane 2 was from a wild-type fetus and the sample in lane 11 was from a heterozygous fetus.*

6.2.4   MCT8 mRNA expression assay

RNA was extracted from frozen placental tissue harvested from male fetuses at E18.5 and reverse transcribed as described in sections 2.2.2 and 2.2.3. MCT8 mRNA expression was compared between placentae from MCT8$^{-/0}$ (n = 6) and MCT8$^{+/0}$ fetuses (n = 11) following the protocol described in section 2.2.5 using primer and probe sequences designed using the human MCT8 sequence and shown in **Table 6-4**.

Table 6-4: *Primer and probe sequences for relative quantification of mouse MCT8 expression by TaqMan PCR.*

Forward Primer	Reverse Primer	Probe	Amplicon length (bp)
GCCCTGCCAATG ATTGCT	CAAAGTAGAAGGCC ACATGGTAGT	CCCATTGCA<u>GG</u>CCT ACTCCGCA	81

Sequences are given in the 5'-3' direction. Underlined base pairs correspond to the boundary between exons 5 and 6.

6.2.5   Stereo-histological assessment of the placenta

Following overnight incubation in paraformaldehyde/PIPES, the placental halves were washed twice with PBS (15 minute washes). They were washed in 70% ethanol for 30 minutes and were stored in 70% ethanol at 4°C until they were transported to Birmingham. The tissue was dehydrated by a 30-minute wash in 70% ethanol followed by three washes in 90% ethanol and three washes in 100% ethanol. The samples were incubated in histoclear overnight and then washed in fresh histoclear for another 60 minutes. Before embedding, the tissues were incubated for 3 hours in paraffin at 58°C and during this incubation paraffin was replaced with fresh paraffin every hour. They were then embedded in fresh paraffin with the cut surface facing the bottom of the mould. Paraffin embedded placentae from two MCT8$^{-/0}$ and two MCT8$^{+/0}$ fetuses from the same litter (749) were exhaustively sectioned to 7 μm-thick sections and mounted onto slides.

Randomly selected sections ranging from the centre to the periphery of the placenta (20 sections per placenta) were stained with Harris Haematoxylin to visualise the nuclei and Eosin to stain the cytoplasm (H&E staining). The sections were incubated in xylene for 5 minutes followed by two 3-minute washes in industrial methylated spirits 99% (IMS) and a 5-minute wash in tap water. They were stained in Haematoxylin for 3 minutes followed by a 2-minute wash in tap water. After a 30-second incubation in acid alcohol (1%), the samples were washed in tap water for 2 minutes and incubated in Scott's tap water for 30 seconds. The sections were stained in eosin for 2 minutes, washed in tap water for 2 minutes and washed twice in IMS for 2 minutes. Following a 2-minute incubation in xylene, the sections were mounted using DPX mountant (all from Surgipath, Peterborough, UK). The sections were photographed using a $2\times$ objective lens and the volume fraction of the labyrinth zone and the junctional zone were estimated following the methodology described in Coan *et al.* (Coan *et al.*, 2004). A grid was superimposed over each section and the total number of points falling on the labyrinth zone, the junctional zone and the whole placenta were counted. The volume fraction of each zone was estimated as the number of points falling on that zone divided by the number of points falling on the placenta. In order to calculate the absolute placental volume, the wet placental weight of each sample was multiplied by 1.05, which is the estimate of the specific gravity of the human placenta (Laga *et al.*, 1973). This value was then multiplied by the volume fraction of each zone in order to get an estimate of the absolute volume of the labyrinth and junctional zone.

6.2.6   Statistical analysis

Differences between the $MCT8^{-/0}$ group and the $MCT8^{+/0}$ group were analysed by unpaired two-tailed Student's t test using the Minitab® statistical software (version 15).

6.3   Results

6.3.1   MCT8 mRNA expression in MCT8$^{-/0}$ and MCT8$^{+/0}$ placentae

The knockdown of MCT8 mRNA expression in the placentae of MCT8-deficient fetuses at E18.5 was confirmed by quantitative TaqMan PCR. MCT8 mRNA expression was reduced by 84% in MCT8$^{-/0}$ placentae compared with MCT8$^{+/0}$ placentae (P<0.01; **Figure 6-4).**
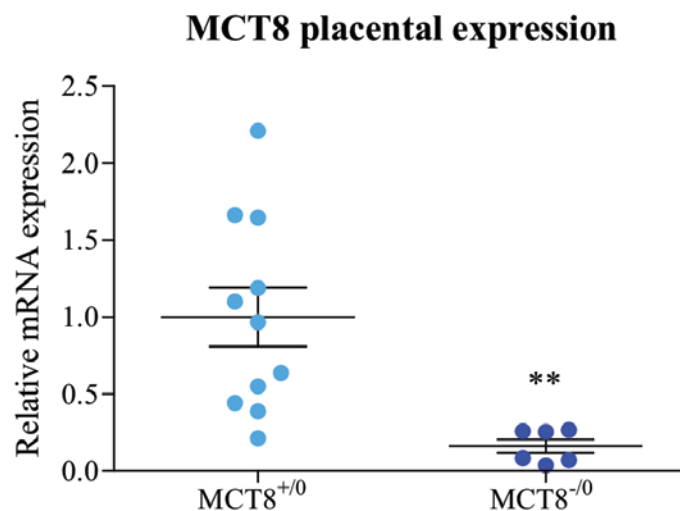
*Figure 6-4: Relative mRNA expression of MCT8 in MCT8$^{+/0}$ (n = 11) and MCT8$^{-/0}$ (n = 6) placentae disected at E18.5. Expression is compared to the mean MCT8 expression in MCT8$^{+/0}$ placentae that was given the arbitrary value of one. Statistically significant differences between groups are indicated by ** (P < 0.01; t test).*

6.3.2   Fetoplacental weights

Individual wet fetal and placental weights of MCT8$^{+/0}$ and MCT8$^{-/0}$ fetuses were compared. Due to the small number of litters (n = 2 for both gestational ages) that had fetuses of both MCT8$^{+/0}$ and MCT8$^{-/0}$ genotypes, it was not possible to compare the

average fetal and placental weights per litter, therefore the analysis was performed on pooled litters.

At E14.5, the average fetal weight of MCT8$^{+/0}$ fetuses was 308.1 mg $\pm$ 33.8 (n = 3; average $\pm$ SEM) and the average fetal weight of MCT8$^{-/0}$ fetuses was 313.1 mg $\pm$ 4.7 (n = 9; **Figure 6-5A**). However, at E18.5, MCT8$^{-/0}$ fetuses (n = 7) were 9% lighter compared with MCT8$^{+/0}$ fetuses (n = 16) ($P < 0.05$; **Figure 6-5B**; average fetal weight $\pm$ SEM: 1273 mg $\pm$ 24.5 and 1397 $\pm$ 28.6 respectively).
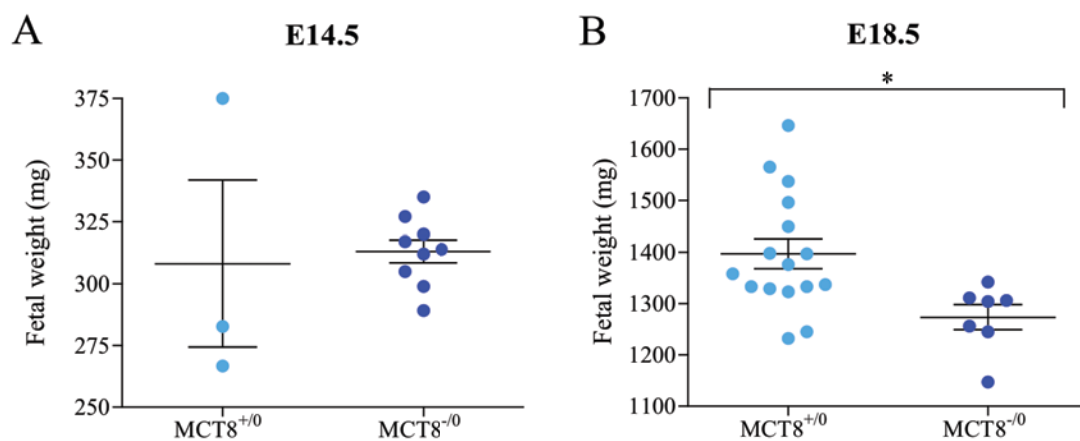
Figure 6-5: *Weights of MCT8$^{+/0}$ and MCT8$^{-/0}$ fetuses at gestational day 14.5 (**A**; E14.5) and at gestational day 18.5 (**B**; E18.5). Points represent weights of individual fetuses and the lines represent the mean fetal weight for each group. Statistically significant differences between groups are indicated by * (P<0.05; t test).*

The placental weights of the two groups were also compared in order to assess whether placental growth was altered as a consequence of knocking down MCT8. The average weight of placentae from MCT8$^{-/0}$ fetuses at E14.5 was 81.3 mg $\pm$ 3.8 (n = 9), whilst the average placenta weight from MCT8$^{+/0}$ fetuses was 67.1 mg $\pm$ 5.7 (n = 3) (NS; **Figure 6-6A**). At E18.5 the average placenta weight of MCT8$^{-/0}$ fetuses (n = 7) was 17% greater compared with the average placental weight from MCT8$^{+/0}$ fetuses (n = 16) ($P <$

0.05; **Figure 6-6B**; average placental weight ± SEM: 90.3 mg ± 6.5 and 77.3 ± 2.8, respectively).
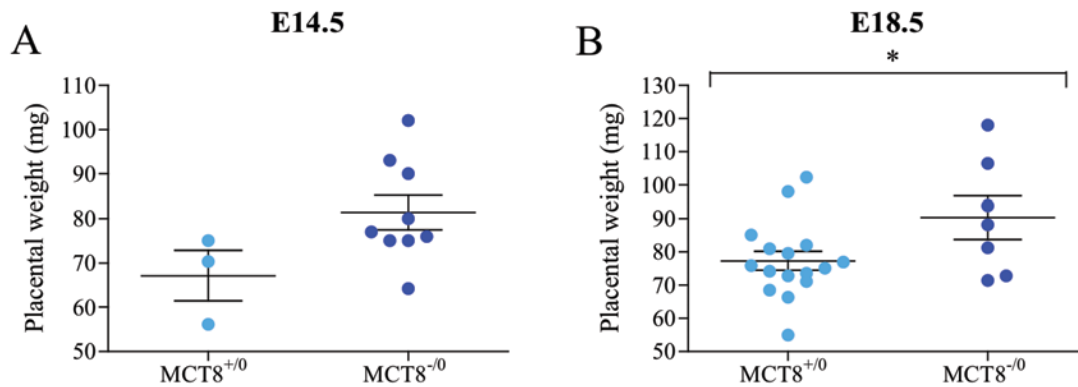
Figure 6-6: *Weights of placentae from MCT8$^{+/0}$ and MCT8$^{-/0}$ fetuses at gestational day 14.5 (**A**; E14.5) and at gestational day 18.5 (**B**; E18.5). Points represent weights of individual samples and the lines represent the mean placental weight for each group. Statistically significant differences between groups are indicated by * (P<0.05; t test).*

Fetal to placental weight ratios in MCT8$^{-/0}$ and MCT8$^{+/0}$ fetuses were calculated to assess placental efficiency (Coan *et al.*, 2008) and it was found that in MCT8$^{-/0}$ fetuses the placenta supported less grams of fetus per gram of placenta compared with MCT8$^{+/0}$ fetuses, indicating that the MCT8$^{-/0}$ placenta is less efficient than the MCT8$^{+/0}$ placenta. The ratio of fetal to placental weight was decreased by 18% at E14.5 (NS; **Figure 6-7A**) and by 28% at E18.5 (P<0.01; **Figure 6-7B**) in MCT8$^{-/0}$ fetuses compared with MCT8$^{+/0}$ fetuses.
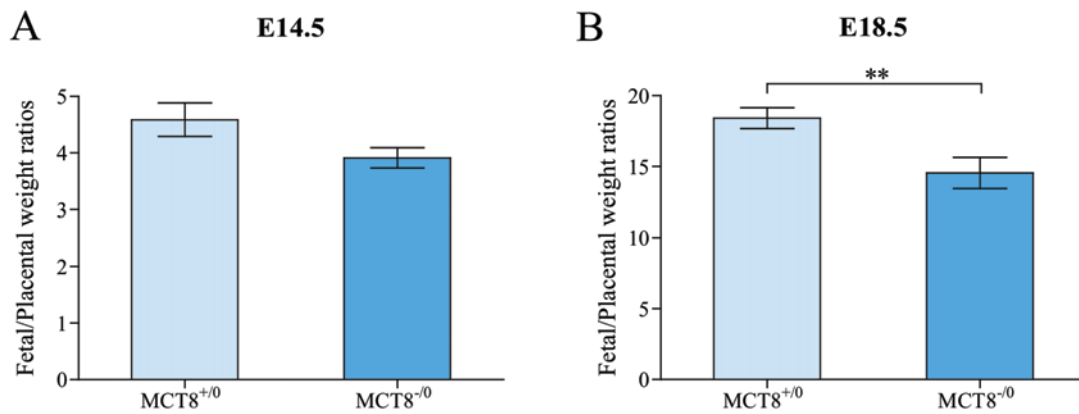
Figure 6-7: *Ratios of fetal/placental weights for MCT8$^{+/0}$ and MCT8$^{-/0}$ fetuses at gestational day 14.5 (**A**; E14.5) and at gestational day 18.5 (**B**; E18.5). Bars represent average ratios for each group +SEM (E14.5: MCT8$^{+/0}$ n = 3, MCT8$^{-/0}$ n = 9; E18.5: MCT8$^{+/0}$ n = 16, MCT8$^{-/0}$ n = 7). Statistically significant differences between groups are indicated by ** (P<0.01; t test).*

6.3.3   Stereo-histological assessment of the placenta

The stereology (**Figure 6-8**) of placentae from two MCT8$^{+/0}$ and two MCT8$^{-/0}$ fetuses obtained from the same litter (litter 749; E18.5) was assessed. The volume fraction of the junctional zone was increased whilst the volume fraction of the labyrinth zone was decreased in placentae from MCT8$^{-/0}$ fetuses compared with placentae from MCT8$^{+/0}$ littermates (**Figure 6-9**). Furthermore, the calculated absolute volume of the junctional zone was 10% greater in the MCT8$^{-/0}$ placentae, whilst the absolute volume of the labyrinth was smaller by 5% (**Table 6-5**). However, these results did not reach statistical significance in the limited sample number that was assessed.
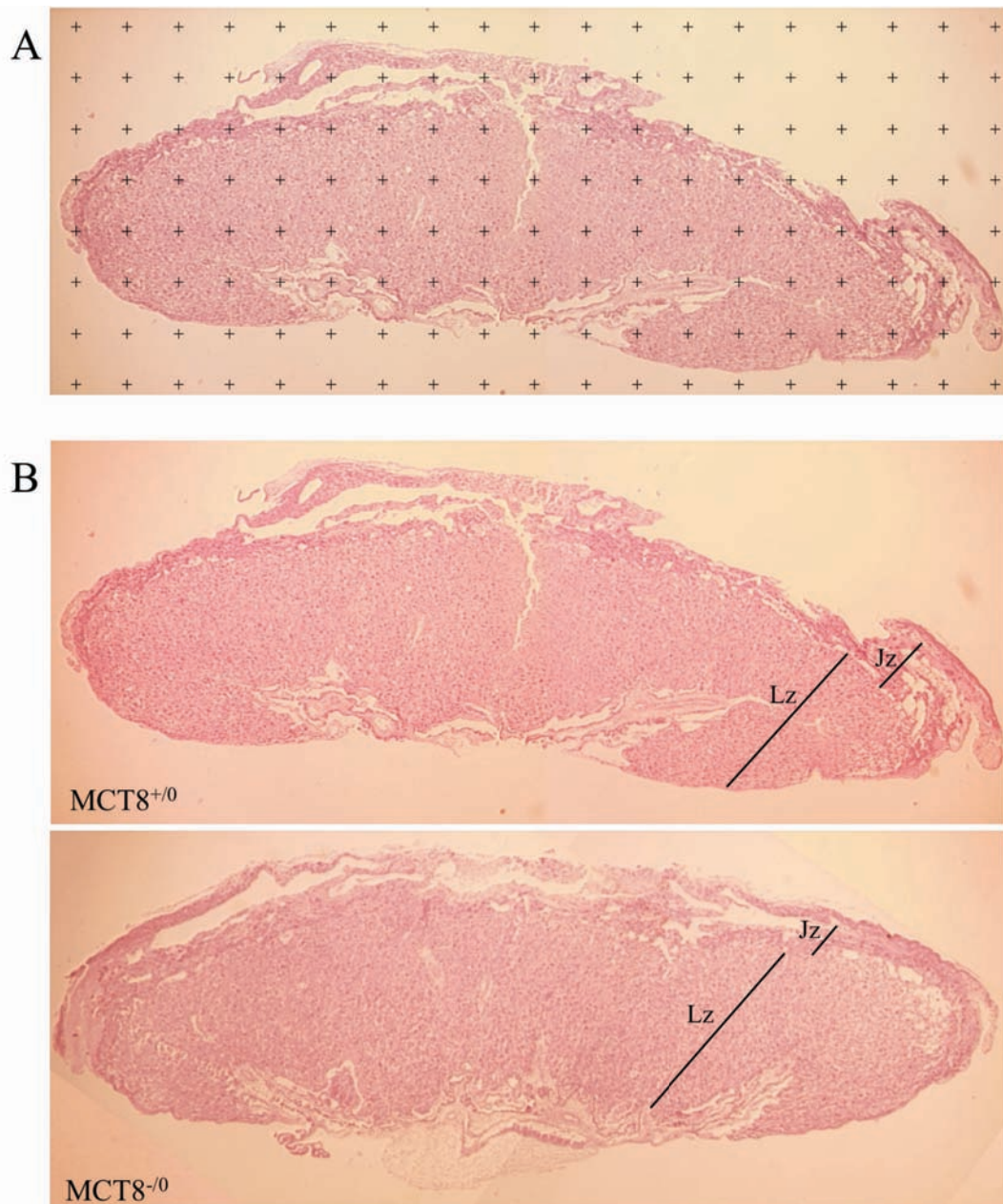
Figure 6-8: *Paraffin sections of E18.5 placentae. The sections were stained with Harris Hematoxylin and Eosin and were viewed under a 2× objective. **(A)** A grid was superimposed over each section and component densities of each zone relative to the placenta were measured by point counting. **(B)** Junctional (Jz) and labyrinth zones (Lz) in representative sections from one wild-type (MCT8+/0) and one knockout (MCT8-/0) placenta.*
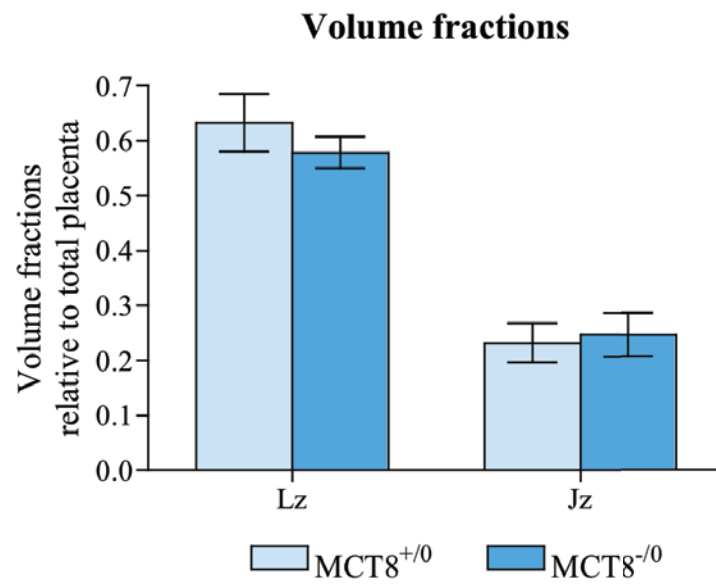
Figure 6-9: *Volume fractions of labyrinth (Lz) and junctional zones (Jz) of MCT8$^{+/0}$ and MCT8$^{-/0}$ placentae. Bars represent the average volume fractions of two samples for each group +SEM.*

Table 6-5: *Absolute volumes (cm^3) of total placenta, Lz and Jz in the MCT8$^{+/0}$ and the MCT8$^{-/0}$ mouse placenta.*

	MCT8$^{+/0}$	**MCT8$^{-/0}$**
Placenta	0.073 ± 0.003	0.076 ± 0.001
Lz	0.046 ± 0.002	0.044 ± 0.003
Jz	0.017 ± 0.003	0.019 ± 0.003

Values are mean volumes obtained from two samples per group ± SEM. Lz, Labyrinth zone; Jz, Junctional zone.

6.4   Discussion and conclusions

6.4.1   Effect of knocking down MCT8 on murine placental development and fetal growth

In this study, the MCT8 knockout mouse model developed by Dr H Heuer's laboratory (Trajkovic *et al.*, 2007) was used and preliminary results were obtained on the potential role of MCT8 in mouse placental development and fetal growth. At E18.5, which is one day before normal delivery, the growth of MCT8$^{-/0}$ fetuses was impaired although placental weight was increased. The fetal to placental weight ratio of MCT8$^{-/0}$ animals was significantly reduced indicating that the MCT8-deficient placenta supports less grams of fetus per gram of placenta when compared with the placenta of normal fetuses and is thus less efficient at sustaining fetal growth (Coan *et al.*, 2008). At E14.5, which is before the onset of fetal TH production (De Felice *et al.*, 2004), there was no significant difference in the weights of MCT8$^{-/0}$ and MCT8$^{+/0}$ fetuses. However, similar trends to E18.5 were observed, with increased placental weight and decreased fetal to placental weight ratios in MCT8-deficient mice. These initial results suggest that although male MCT8-deficient mice have heavier placentae compared with male wild-type fetuses, their placentae are less efficient. This appears to result in impaired fetal growth at late gestation (E18.5).

The effect of MCT8 knockdown on fetoplacental weights suggests that the lack of MCT8 may affect placental morphology and structure. There were no profound changes in the cellular structure of the placenta when comparing MCT8$^{-/0}$ and MCT8$^{+/0}$ placentae. However, quantification of the volume fractions of each zone revealed that whilst the volume fraction of the junctional zone was increased, the volume fraction of the labyrinth

zone was decreased in the MCT8$^{-/0}$ compared with MCT8$^{+/0}$ placentae. This observation persisted when absolute volumes were calculated, suggesting that the volume of the junctional zone is greater in the knockout placenta, whilst the labyrinth zone is smaller. These results were based on a small sample number and did not reach statistical significance, however, if confirmed in more samples, they suggest that MCT8 knockdown may alter the volumes of the placental components. The labyrinth zone of the placenta is the region responsible for maternofetal nutrient exchange (Watson and Cross, 2005). Therefore, a reduction in the volume of the labyrinth may be associated with reduced transport capacity of MCT8$^{-/0}$ placenta and may contribute to impaired growth of MCT8$^{-/0}$ fetuses. It is possible that the increased weight of the placenta of knockout fetuses reflects an attempt to compensate for the reduced volume of the labyrinth zone. However, these initial results suggest that the even though the knockout placenta is larger compared with the wild-type, the volume of the labyrinth zone is smaller.

6.4.2   Study limitations and future work

This study provided important information about the potential role of MCT8 in mouse placental development. However, there are limitations to this work mostly due to the small sample number that was assessed. Future work needs to be carried out in order to confirm these results in larger sample groups. Assessment of placental and fetal weights from more litters will allow for analysis of the results per litter and can give a more accurate estimate on the effect of MCT8 knockout on placental and fetal weights. The results obtained from this sample cohort suggest that the growth of MCT8-null fetuses is significantly impaired at E18.5 but not at E14.5. It would be useful to assess placental and fetal weights across a broader range of gestational ages, including E16.5, in order to determine the gestational age at which fetal growth is most compromised.

Stereohistological assessment of the placenta can provide information about the placental zones that may be affected by the lack of MCT8 and thus identify developmental pathways that may be compromised. The initial assessment presented here suggests that the volume fraction of the labyrinth zone is reduced in MCT8-null placentae, however this study needs to be expanded in order to include more samples from E18.5 and from earlier gestations, which could help to identify when placental architecture is first affected. Furthermore, it would be useful to analyse such sections further in order to determine the surface areas of the maternal blood spaces and the fetal capillaries and measure the thickness of the interhemal membrane, which constitutes the barrier between the maternal and the fetal circulation in the labyrinth zone of the mouse placenta (Coan *et al.*, 2004). This information could be used in order to estimate and compare the theoretical diffusing capacity of the MCT8$^{-/0}$ and the MCT8$^{+/0}$ placenta (Coan *et al.*, 2004).

Lack of MCT8 may result in impaired transplacental transport of THs. In the placenta, this could have implications for both placental development, that is known to be responsive to THs (Kilby *et al.*, 2005), and for transplacental transport of maternal THs to the fetal circulation where they can reach TH-responsive fetal tissues, such as the developing brain (Chan and Kilby, 2000). In order to characterise transplacental transport of THs in MCT8-null mice, radioimmunoassays could be used to measure the concentrations of THs in the maternal circulation and in the fetus (Morreale de Escobar *et al.*, 1985). Previous studies in MCT8-null mice have shown that TH availability varies between tissues depending on the tissue-specific expression of other TH transporters and on the activity of deiodinases. MCT8 knockout results in a hypothyroid state of the brain, where MCT8 appears to play a crucial role for TH uptake, whilst the liver and the kidneys are hyperthyroid (Dumitrescu *et al.*, 2006, Trajkovic *et al.*, 2007, Trajkovic-Arsic *et al.*,

2009). Therefore, quantification and localisation of the expression of MCT8 and other TH transporters, and of deiodinase enzymes, in the mouse placenta across gestation would aid the understanding of the TH transport system in the mouse placenta. These further studies would greatly help to determine the importance of MCT8 for transplacental TH transport in mice and investigate the possibility that changes in the expression of other TH transporters and in the activity of deiodinase enzymes may partly compensate for the absence of MCT8. However, *in vitro*, MCT8 appears to affect human placental cells through both T_3-dependent and T_3-independent mechanisms, as discussed in Chapters 4 and 5. It is therefore possible that the observed changes in the fetoplacental weights of MCT8-deficient fetuses are not associated with changes in transplacental transport of THs but with the transport of another compound important for development.

6.4.3   Conclusions

MCT8 knockout mouse models are useful tools to assess the role of MCT8 in placental development and fetal growth. The preliminary results presented here suggest that lack of MCT8 adversely affects fetoplacental growth. However, it is important to recognise that the mechanisms that regulate fetoplacental development may differ in mice from humans. This issue is highlighted by the absence of a severe neurological phenotype in MCT8-deficient mice in contrast with that observed in patients affected by MCT8 mutations. Furthermore, TH transport is regulated by many TH transporters and the expression of these proteins in the mouse placenta has not yet been defined. Even though the extrapolation of results from animal studies to humans needs to be done with caution, such studies can help to elucidate the mechanisms that regulate TH transport across the placenta and may provide further insight on whether MCT8 transports compounds other than THs that may be important for fetoplacental development.

Chapter 7: Final Discussion and Conclusions

7.1 TH transporters in the human placenta and transplacental TH transport

Transplacental transport of THs is of topical interest due to the importance of THs for fetal development (Haddow *et al.*, 1999, Morreale de Escobar *et al.*, 2004, Pop *et al.*, 2003). In this thesis, the ontogenic pattern of the TH transporters that have been reported in the human placenta was described. The expression of a range of TH transporters from early gestation is consistent with the hypothesis that transplacental transport of maternal THs can occur from early in pregnancy and until term. Furthermore, the expression of the TH transporters studied peaked towards term, suggesting that the supply of maternal THs may be necessary even after the onset of fetal TH production.

Localisation of the TH transporters in specific human placental cells provided additional information on the TH transport capability of these cell types. The expression of TH transporters in the ST is particularly important, since these cells constitute the trophoblast layer that primarily regulates transplacental transport. It was previously shown that MCT8 is expressed in the ST throughout gestation (Chan *et al.*, 2006). The LAT1/4F2hc complex and OATP4A1 were previously localised in the ST of third trimester placentae (Okamoto *et al.*, 2002, Ritchie and Taylor, 2001, Sato *et al.*, 2003). The results presented in this thesis demonstrate that MCT10, OATP1A2 and OATP4A1 are also expressed in the ST of the placenta both in the first and in the third trimester. Taken together, these data demonstrate the presence of a range of TH transporters in the ST of the human placenta, where they can regulate transplacental TH transport, both in early and late pregnancy. In addition to the importance of the THs in fetal development, there is increasing evidence that THs play an important role in the development of the placenta (Barber *et al.*, 2005, Laoag-Fernandez *et al.*, 2004, Maruo *et al.*, 1991, Oki *et al.*,

2004). The expression of TH transporters in CTs and EVTs is consistent with a role for THs in the regulation of trophoblast differentiation and function. MCT8 (Chan *et al.*, 2006), MCT10 and OATP1A2 are found in CT cells, whilst EVTs express MCT8, MCT10, OATP1A2 and OATP4A1 (Loubière *et al.*, 2010).

Although the characterisation of TH transporter expression in human trophoblast cells across gestation is valuable for the understanding of the regulation of transplacental TH transport, this information is not sufficient to provide a full picture of TH transport through the placenta and into the fetal circulation. The TH transporters described have different affinities for THs and, with the exception of MCT8, are known to transport more substrates in addition to THs (see **Table 1-1**). Therefore, their contribution towards transplacental TH transport will depend on both their affinity for THs and on the circulating concentration of other substrates that may competitively inhibit TH transport. Future investigations of TH transport across the placenta are therefore necessary. Vesicles generated from the apical (microvillous; maternal facing) or the basal (fetal facing) plasma membranes of the ST would be a useful system to study transplacental TH transport (Glazier and Sibley, 2006). The contribution of each transporter to overall TH transport could be elucidated using inhibitors, such as tryptophan for MCT10 (Friesema *et al.*, 2008, Kim *et al.*, 2002), 2-amino-bicycloheptane-2-carboxylic acid for system L (Ritchie *et al.*, 2003, Ritchie and Taylor, 2001) and bile acids or other amphipathic organic compounds for OATPs (Hagenbuch, 2007).

It is currently unknown whether changes in transplacental transport of THs are associated with pregnancy complications, such as IUGR. The fetal circulating concentrations of free T_4 and free T_3 are reduced in severe, early onset IUGR pregnancies (Kilby *et al.*, 1998). In the third trimester, THs in the fetal circulation are of both maternal

and fetal origin (see **Figure 1-9**). It is therefore difficult to speculate whether the reduced TH concentrations in fetuses affected by IUGR, are due to reduced transplacental transport of maternal THs or reduced fetal TH synthesis. However, the increased protein expression of MCT8 in the placenta in severe IUGR pregnancies suggest that transplacental TH transport may be altered in IUGR pregnancies. IUGR has been associated with reduced transplacental transport of amino acids (Sibley, 2009). Jansson *et al.* have demonstrated that the transport of leucine and lysine, which is facilitated by the system L is reduced in IUGR placentae (Jansson *et al.*, 1998). It is therefore possible that the protein expression or activity of system L is altered with IUGR and this may impact on transplacental TH transport in addition to amino acid transport.

In this thesis, primary cultures of CTs from third trimester placentae from normal pregnancies or non-severe IUGR pregnancies that syncytialised *in vitro* were used to assess transport of T_3 and we found that there was increased intracellular accumulation of T_3 within IUGR compared with normal CTs. This was accompanied by an increase in the expression of MCT8 in IUGR compared with normal CTs, whilst MCT10 expression was not affected in these samples. If these *in vitro* results are representative of the situation in the ST *in vivo*, they indicate that maternal T_3 may be retained in the ST instead of being effluxed towards the fetal capillaries thus contributing to the decreased concentration of free T_3 in the fetal circulation with IUGR. It is likely that a combination of factors, including increased expression of MCT8, increased individual volume of IUGR CTs due to increased syncytialisation (Crocker *et al.*, 2004, Newhouse *et al.*, 2007) and increased expression of intracellular T_3-binding proteins, such as TRs (Kilby *et al.*, 1998) contribute to the accumulation of T_3 intracellularly *in vitro*. Furthermore, it is necessary to investigate the transport of T_4 by normal and IUGR CTs and/or vesicle preparations, since

T_4 is considered the TH hormone that is predominantly transported from the maternal to the fetal circulation (Chan *et al.*, 2009). The investigation of TH transport in CTs from severe IUGR pregnancies is difficult due to the sparsity of gestationally-matched normal controls. However, assessment of TH transport in such samples would provide a picture of placental TH transport in pregnancies complicated by severe IUGR that are associated with increased risk for adverse fetal outcome (Gaffney *et al.*, 1994, Kok *et al.*, 1998).

Our *ex vivo* results on the expression of MCT8 in placentae from normal and severe IUGR pregnancies were obtained using villous placental biopsies, which consist of different cell types. Therefore, they do not provide any information on the expression level of MCT8 in specific cell types i.e. CTs or ST. Furthermore, there is no information regarding the expression of TH transporters in the apical or basal membranes of the ST in normal and IUGR pregnancies. It has been previously reported that in many cases, changes in the activity of transporters in the ST in IUGR pregnancies are specific to either the apical or the basal ST membrane (Sibley, 2009). Such examples include system A that is decreased in the apical but not in the basal membrane with IUGR (Glazier *et al.*, 1997, Jansson *et al.*, 2002) and the lactate transporter that is decreased in the basal but not in the apical membrane as shown in experiments with preparations from the basal or the apical membrane of the ST (Settle *et al.*, 2006). Nutrients, including THs have to cross both these membranes in order to reach the fetal capillaries. The identification of the transporters that facilitate the transport of THs across the basal and the apical membranes of the ST would therefore contribute to the understanding of the mechanisms that control transplacental TH transport. Vesicle preparations from the apical or the basal membranes from normal or IUGR placentae could be used to assess the activity of different TH

transporters and confirm whether the observed differences in expression affect transplacental transport.

Although the results described here provide valuable information about transplacental TH transport in the third trimester in uncomplicated pregnancies and in pregnancies complicated by IUGR, there are no data available on the transport of THs in IUGR pregnancies early in gestation, which is a crucial period for both fetal and placental development. The realisation of such studies in the human placenta is hindered by the fact that IUGR is not diagnosed until later in gestation. However, animal studies could be performed to investigate transplacental TH transport early in pregnancy. IUGR animal models have been previously utilised to investigate fetoplacental growth (Vuguin, 2007). The advantage of such studies is that they would allow *in vivo* assessment of TH transport. However, the extrapolation of findings from animal models to humans is not always straightforward, especially because placentation differs between mammalian species.

THs are necessary for normal development of both the fetus and the palcenta. Therefore, the supply of optimal TH levels from the maternal circulation to the placenta and the fetus is critical in pregnancy. IUGR is associated with increased risk for adverse fetal outcomes including increased risk for neurodevelopmental disorders (Kok *et al.*, 1998). Since THs are known to play a major role in the development of the CNS (Haddow *et al.*, 1999, Pop *et al.*, 2003, Pop *et al.*, 1999), abnormal transplacental TH transport in IUGR pregnancies may contribute to the increased risk for neurodevelopmental delay. The results presented in this thesis provide evidence that transplacental TH transport may be altered in IUGR pregnancies.

7.2   T₃-responsiveness of trophoblast cells

In addition to their importance for fetal development, there is increasing evidence that THs affect the development of the placenta, particularly during early pregnancy. It has been previously reported that T_3 increases invasion by EVTs (Oki *et al.*, 2004). EVT invasion in the maternal endometrium and transformation of maternal spiral arteries by EVTs are important events that occur early in pregnancy and are essential for normal placentation and for the establishment of unobstructed blood flow from the maternal circulation to the placenta (Huppertz and Peeters, 2005). Due to difficulties in accessing placental tissue from early pregnancy, we have used the cell line SGHPL-4 as an EVT cell model and we have confirmed the promoting effect of T_3 on invasion using this cell line. In addition, we found that T_3 results in a mild rise in the proliferation of these cells but, unlike findings in primary EVTs (Laoag-Fernandez *et al.*, 2004), does not affect apoptosis. Most of the effects of T_3 are mediated via TRs that regulate gene expression. Many genes that are regulated by TRs have been identified in other cell types and they include Cx43, which encodes a gap junction protein that has a role in intercellular communication (Stock and Sies, 2000), and ME1, a cytosolic enzyme (Gonzalez-Manchon *et al.*, 1997). However, TR-mediated regulation of genes is cell type-dependent (Yen, 2001) and according to our findings, T_3 does not affect the expression of either Cx43 or ME1 in SGHPL-4s. Further experiments are required to identify the genes that are regulated by T_3 and result in increased SGHPL-4 invasion. Such a candidate could be furin, a protease that is upregulated by T_3 as demonstrated in a hepatocellular carcinoma cell line (Chen *et al.*, 2008b) and which promotes invasion by the EVT-like cell line, HTR8 (Zhou *et al.*, 2009).

In contrast to EVT-like cells, we found that T_3 had no effect on CTs from third trimester normal placentae, in terms of CT survival, apoptosis or syncytialisation assessed by hCG secretion. This is in accordance with previous data that have shown that whilst treatment with T_3 promotes the endocrine activity of placental explants from early pregnancy, as demonstrated by increased secretion of progesterone, estradiol-17β, hCGα, hCGβ, hCG and hPL, it does not affect the endocrine activity of explants from term placenta (Maruo *et al.*, 1991). Interestingly, we found that in contrast with CTs from normal placenta, IUGR CTs were responsive to T_3 treatment, as demonstrated by decreased survival, and increased apoptosis in response to T_3. IUGR has been associated with increased apoptosis (Endo *et al.*, 2005, Ishihara *et al.*, 2002, Smith *et al.*, 1997) and increased syncytialisation (Crocker *et al.*, 2004, Newhouse *et al.*, 2007). However, the findings presented in this thesis demonstrate for the first time that T_3 can adversely affect the survival and increase apoptosis of IUGR CTs and they raise the possibility that abnormally high levels of THs locally within the placenta may directly impact upon trophoblasts and contribute to the pathogenesis of IUGR in vulnerable pregnancies.

The mechanisms that result in the differential T_3 responsiveness of normal and IUGR CTs are unclear at present. Increased intracellular accumulation of T_3 within IUGR CTs, compared with normal CTs is likely to contribute to the increased responsiveness of IUGR CTs to treatment with T_3. Previously, it was reported that the protein, but not the mRNA, expression of the TR isoforms TRα1, TRα2 and TRβ1 was increased in sections obtained from IUGR placentae, compared with normal controls (Kilby *et al.*, 1998). In the experiments with primary cultures of IUGR and normal CTs presented in this thesis, the mRNA expression of TRs was assessed and no differences were found between the IUGR and the normal group. However, the protein expression of TRs in these samples has not

been assessed and, similar to the previous study by our group (Kilby *et al.*, 1998), mRNA expression may not correlate with protein expression. Increased accumulation of T_3 within IUGR CTs, in conjunction with increased expression of TRs may cause increased activation of TH-responsive genes thus resulting in the altered responsiveness of IUGR CTs. In addition, the possibility that T_3 affects IUGR CTs via non-genomic mechanisms has to be considered. It has been demonstrated that THs influence the proliferation of tumour cells via binding to a cell surface TR located on integrin $\alpha V \beta 3$ (Davis *et al.*, 2008). Although T_4 is the predominant ligand for this receptor, T_3 can also bind to this TR (Davis *et al.*, 2008). In addition, T_3 can bind to TRs located in the cytoplasm (Davis *et al.*, 2008). T_3 binding to extranuclear TRs initiates MAP kinase pathways that can in turn influence cell behaviour. Furthermore, T_3 can bind to mitochondria-resident TRs and thus initiate the expression of mitochondrial genes (Wrutniak-Cabello *et al.*, 2001). Unlike findings in CNS cell types (Bianco *et al.*, 2002), treatment of term CTs with 10 nM or less T_3 does not alter the expression of the deiodinase enzymes, D2 and D3 (Chan *et al.*, 2003). Furthermore, no significant changes were observed in D2 and D3 mRNA expression and activity in biopsies from normal compared with IUGR placentae (Chan *et al.*, 2003). Taken together these findings suggest that CTs cannot modulate intracellular T_3 concentration through alterations in D2 and D3 activities, thus rendering IUGR CTs more vulnerable to the increased accumulation of T_3 intracellularly that was demonstrated herein.

Further experiments are necessary to elucidate the mechanisms that are involved in the responsiveness of IUGR, but not normal CTs, to T_3. The present findings have demonstrated that increased accumulation of T_3 intracellularly is associated with increased MCT8 protein expression, whilst the expression of MCT10 is not altered.

Assessment of the protein expression of the other TH transporters expressed in the human placenta: system L, OATP1A2 and OATP4A1 within CTs is necessary to better understand the mechanisms that result in increased net T_3 uptake by IUGR CTs. The involvement of mechanisms in addition to increased MCT8 expression in the increased net T_3 uptake in IUGR CTs is indicated by uptake studies in trophoblast cells transfected with human MCT8. These experiments demonstrated that although MCT8 overexpression increased initial T_3 uptake, net T_3 uptake after 30 minutes of incubation with $[^{125}I]$-T_3 was similar in cells transfected with MCT8 or VO, thus indicating that overexpression of MCT8 alone cannot explain the intracellular accumulation of T_3 in IUGR CTs. In contrast, co-transfection with CRYM, a cytoplasmic protein that binds to T_3 intracellularly, resulted in increased net T_3 uptake. It is therefore likely that increased expression of T_3 binding proteins, such as TRs, may contribute to the intracellular accumulation of T_3 within IUGR CTs. In order to conclude whether increased T_3 binding to TRs is contributing to the increased accumulation of intracellular T_3 and increased T_3 sensitivity of IUGR CTs, the previously reported increase in the expression of TRs in IUGR placental biopsies (Kilby *et al.*, 1998), should be confirmed in the CT-culture model. Further assessment of the effects of T_3 on IUGR CTs would involve the identification of the pathways that are activated in IUGR CTs in response to T_3 treatment. One possible mechanism could be that T_3 increases cell metabolism and therefore oxygen consumption (Yen, 2001). Although oxygen consumption is essential for energy expenditure and cell metabolism, it also results in the generation of reactive oxygen intermediates and free radicals, which are associated with oxidative stress and cell damage. Oxidative stress is associated with IUGR and it is likely that IUGR CTs are more sensitive to oxidative damage than normal CTs thus resulting in decreased cell survival and increased apoptosis of IUGR CTs in response to T_3. However, it is currently difficult

to speculate whether the altered responsiveness of IUGR CTs to T_3 is a contributing factor or an effect of the pathology associated with these pregnancies.

The impact of THs on trophoblast cells is of great clinical interest, especially because abnormal maternal TH status is a risk factor for pregnancy complications associated with malplacentation, such as IUGR. Assessment of the impact that changes in TH availability have on trophoblast cells *in vitro* would aid the understanding of the effects of abnormal maternal TH status on placental development *in vivo* and could supply important information relevant to the clinical debate on the screening of maternal thyroid function during pregnancy and even more so in women who previously had pregnancies complicated by IUGR.

7.3   The role of MCT8 in trophoblast cells

The expression of MCT8 in EVTs, CTs and ST of the human placenta throughout gestation indicates that MCT8 may play a role in the development of the placenta (Chan *et al.*, 2006). Furthermore, the finding that MCT8 expression is increased in placentae from IUGR pregnancies suggests that MCT8 may be associated with the malplacentation associated with IUGR. In order to investigate the role of MCT8 in different trophoblast types, MCT8 expression was silenced or upregulated in the invasive EVT-like cell line, SGHPL-4 and in primary cultures of CTs from term placentae that spontaneously syncytialise *in vitro*.

As discussed in the previous section, T_3 promotes invasion by primary EVTs (Oki *et al.*, 2004) *in vitro*. In SGHPL-4s, T_3 treatment results in an increasing trend in invasion, which was not statistically significant. However, when SGHPL-4s were transfected with MCT8 T_3 had a statistically significant pro-invasive effect on these cells, consistent with a role for MCT8 in the facilitation of the effects of T_3 on trophoblast cells. This finding indicates that TH transporters, and particularly MCT8, are likely to regulate the effect of T_3 on EVT invasion. The mechanisms that are involved in the T_3-mediated effect on EVT invasion *in vitro* are not completely understood. However, they may include upregulation of the activity of MMPs, degradative enzymes that are associated with EVT invasion in the maternal decidua, as demonstrated in a study by Oki *et al.*, where T_3 increased the expression of MMP-2 and MMP-3 in primary EVTs (Oki *et al.*, 2004).

In addition, it has been demonstrated that T_3 may affect EVT invasion indirectly by reducing EVT apoptosis (Laoag-Fernandez *et al.*, 2004). We therefore investigated the possibility that the MCT8-facilitated effect of T_3 on SGHPL-4 invasion may be mediated

indirectly via changes in cell proliferation, apoptosis or necrosis and thus cell number. In contrast to previous results in the choriocarcinoma cell line JEG-3 (James *et al.*, 2009), MCT8 did not have an effect on SGHPL-4 cell number, indicating that the increase in SGHPL-4 invasion following MCT8 transfection in the presence of T_3 was not mediated via changes in cell number. However, when the endogenous expression of MCT8 was silenced, we did observe a mild decrease of caspase 3/7 activity with no overall effect on cell number as assessed by the MTT assay. In other cell types, such as JEG-3 and N-Tera2 cells, MCT8 did not affect cell apoptosis (James *et al.*, 2009). Similar to the previous report on the effect of MCT8 on cell proliferation (James *et al.*, 2009), the decrease of caspase 3/7 activity following MCT8 silencing in SGHPL-4s was independent of T_3 treatment, suggesting that MCT8 may facilitate the transport of another substrate that mediates this effect. The physiological significance of these findings is unclear, however it should be noted that in contrast to JEG-3 choriocarcinoma-derived cell lines that have a high proliferation rate, SGHPL-4s are derived from EVTs that are cells with an invasive phenotype and with reduced proliferative capacity. The differences in the phenotype of these cells, are likely responsible for the different effects of MCT8 on JEG-3 and SGHPL-4 proliferation.

In contrast to its effects on first trimester trophoblasts, T_3 does not affect the endocrine activity (Maruo *et al.*, 1991) or the cell viability (Barber *et al.*, 2005) of trophoblasts from term placentae. This was confirmed in our experiments with primary CTs from normal term placentae. However, the expression of MCT8 in the human placenta increased with advancing gestation and reached a peak towards term. Although this change may be associated with increased fetal demand for THs, MCT8 may in addition play a role in the regulation of trophoblast behaviour and function in the third

trimester. Modulation of the expression of MCT8 in primary CTs from term placentae demonstrated that MCT8 adversely affects the survival of CTs as assessed by MTT. However, this effect was not mediated via increased activation of the apoptotic caspase cascade or via increased cell necrosis, indicating that MCT8 may affect cell viability or cell metabolic activity via another pathway. In our laboratory, we have recently confirmed that MCT8 affects CT survival by counting the number of nuclei in CT cultures.

Similar to the effect observed previously on JEG-3 proliferation (James *et al.*, 2009), the effect of MCT8 on CT viability was observed both in the absence and in the presence of T_3 indicating that it is not mediated via changes in T_3 transport. The finding that MCT8 has T_3-independect actions in multiple cell types that has been presented both in this thesis and in a study by James *et al.* (James *et al.*, 2009) raises the possibility that MCT8 transports another compound responsible for the observed effects on cell viability and proliferation, which has to be investigated further. Alternatively, MCT8 may affect the pathways that regulate cell survival and proliferation independently of its transporter function. One such possibility could be that even though MCT8 does not transport amino acids, as demonstrated by Friesema *et al.* (Friesema *et al.*, 2003), it may function as an amino acid transceptor, a receptor that is structurally related to a transporter. Amino acid transceptors have a role in sensing extracellular and/or intracellular amino acid availability and activating intracellular pathways as a response. In cases of amino acid deficiency, transceptors are thought to play a role in upregulating proteins that are involved in the transport of deficient amino acids (Goberdhan *et al.*, 2009, Taylor, 2009). Furthermore, signalling by amino acid transceptors may be one of the mechanisms leading to the activation of the mammalian target of rapamycin (mTOR), a key regulator of energy homeostasis, cell physiology and growth (Goberdhan *et al.*, 2009). So far,

SNAT2, a member of the sodium-coupled amino acid transporter (SNAT) family, (Taylor, 2009) and PATH, a member of the proton-assisted amino acid transporter (PAT) family, (Goberdhan *et al.*, 2009) have been identified as amino acid transceptors. It would therefore be useful to address the possibility that MCT8 may function as a transceptor in future studies.

Although the mechanisms that mediate the effect of MCT8 on CT survival are unclear at present, reduced cell survival may adversely affect placental development and growth. As discussed in the previous section, IUGR is associated with increased expression of MCT8 in the placenta. We can therefore speculate that the increased expression of MCT8 may reduce CT viability *in vivo* and thus contribute to the smaller placental size observed in IUGR pregnancies. In contrast, the lack of a change in the secretion of hCG following changes in MCT8 expression suggests that MCT8 is not implicated in the regulation of syncytialisation of term CTs.

Assessment of T_3 uptake in SGHPL-4s or primary CTs following silencing or upregulation of MCT8 expression revealed similar results in both cell types. Silencing of endogenous MCT8 expression decreased T_3 uptake, thus highlighting the TH transport activity of MCT8 in trophoblasts. However, there was significant residual T_3 transport activity even after MCT8 silencing indicating the activity of other TH transporters in addition to MCT8 in these cells. Even though there was significant residual T_3 uptake in trophoblasts following silencing of endogenous MCT8, accumulation of T_3 in these cells was significantly lower than that in control cells even after the longest incubation period tested (30 minutes). Intracellular T_3 accumulation in SGHPL-4s transfected with MCT8 siRNA was lower compared with controls even after co-transfection with CRYM, a cytoplasmic protein that binds to T_3 intracellularly and thus prevents its efflux. These

findings highlight the importance of MCT8 for T_3 transport in these cells and indicate that the other TH transporters present in these cells cannot compensate for the lack of MCT8. Upregulation of MCT8 expression resulted in increased initial T_3 uptake, however it did not affect the net T_3 uptake observed following a 30-minute incubation period with $[^{125}I]$-T_3. Prevention of T_3 efflux by co-transfection with CRYM in SGHPL-4s resulted in increased accumulation of T_3 intracellularly that was maintained after a 30-minute incubation period. These findings suggest that increased T_3 efflux may compensate for the initial increase in T_3 uptake in cells overexpressing MCT8 and may thus maintain the basal intracellular T_3 concentration in the absence of unoccupied intracellular T_3-binding sites.

The importance of MCT8 for the development of the placenta is further highlighted by initial results on the fetal and placental growth of MCT8-deficient mice. According to our findings, MCT8-null fetuses are lighter than their wild-type littermates, whilst their placentae are heavier. This results in decreased fetal to placental weight ratios, indicating that the MCT8-deficient placenta can support less grams of fetus and thus placental efficiency is reduced. The expression of MCT8 in the mouse placenta has not been characterised to date. It is therefore difficult to speculate about the cell types that may be affected by the lack of MCT8. However, initial assessment of the volumes of placental components suggests that the volume of the labyrinth zone, which is responsible for transplacental transport, is reduced in MCT8-null fetuses compared with wild-type littermates, whilst the volume of the junctional zone is increased. A reduction in the volume of the labyrinth zone may therefore reduce nutrient transfer from the maternal to the fetal circulation and thus result in fetal growth restriction in MCT8 knockout mice.

However, it has to be noted that, in humans, MCT8 mutations have not been associated with reduced birth weight.

The results presented in this thesis demonstrate that MCT8 has a role in both transplacental transport of THs and in placental development. Further studies are necessary to better define the role of MCT8 in trophoblasts. The adverse effect of MCT8 on the viability of term CTs suggests that MCT8 may have a role in the regulation of trophoblast cell number and therefore placental size. It would therefore be interesting to assess the role of MCT8 in primary CTs from first trimester placentae, which are the proliferative cells of the placenta that differentiate to EVTs or fuse with the ST depending on the chemical and hormonal signals they receive (Lunghi *et al.*, 2007). An adverse effect of MCT8 on the viability of first trimester CTs would provide further evidence that increased MCT8 expression may be implicated in the reduced placental size in IUGR pregnancies. Furthermore, additional studies are required to elucidate the mechanisms that are involved in mediating the adverse effect of MCT8 on CT viability and particularly to investigate the possibility raised from the results presented in this thesis and from previous results (James *et al.*, 2009) that MCT8 transports another compound in addition to T_3. Furthermore, the MCT8-null mouse model provides a useful tool for further investigations of the role of MCT8 in the transplacental transport of THs and possibly other compounds and in the development of the placenta.

7.4   Conclusions

The findings presented in this thesis indicate the presence of a range of TH transporters in the human placenta throughout gestation thus composing an efficient system for transplacental TH transport that ensures adequate supply of maternal THs to both the developing fetus and the placenta. Our findings suggest that IUGR, a pregnancy complication with increased risk of adverse fetal outcome, is associated with changes in both TH transport and TH action within trophoblast cells. These observations provide further insight into the factors that are involved in the malplacentation associated with IUGR. Furthermore, they are highly relevant to the clinical debate concerning the advantage of universal screening for thyroid dysfunction in women during pregnancy. In this thesis, the role of the TH transporter MCT8 in transplacental TH transport and in the modulation of trophoblast behaviour was further investigated. It was demonstrated that even though there is a range of TH transporters expressed in trophoblast cells, MCT8 plays an essential role in TH transport by trophoblasts. Furthermore, the findings presented here indicate that MCT8 affects the behaviour and survival of trophoblast cells via both T_3-dependent and T_3-independent mechanisms thus suggesting that MCT8 has another function in addition to TH transport. Further investigation of the role of MCT8 in placental development is warranted, particularly in light of the finding that the expression of MCT8 is increased in placentae from pregnancies complicated by IUGR.

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Publications resulting from this thesis

<u>Vasilopoulou, E.</u>, Loubière, L.S., Martín-Santos, A., McCabe, C.J., Franklyn, J.A., Kilby, M.D., Chan, S-Y. (2010) Differential T_3 responsiveness and transport by human cytotrophoblasts from normal and growth-restricted pregnancies. **J. Clin. Endocrinol. Metab.** In press.

Loubière, L.S., <u>Vasilopoulou, E.</u>, Bulmer, J.N., Taylor, P.M., Stieger, B., Verrey, F., McCabe, C.J., Franklyn, J.A., Kilby, M.D. and Chan, S. (2010) Expression of thyroid hormone transporters in the human placenta and changes associated with intrauterine growth restriction. **Placenta***, 31: (4): 295-304.

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Conference Proceedings

<u>Vasilopoulou E</u>, Loubière LS, McCabe CJ, Franklyn JA, H Heuer, M Trajkovic, Kilby MD and Chan SY. The thyroid hormone transporter MCT8 affects human and mouse fetoplacental development. Poster presentation at the **British Thyroid Association Meeting**, November 2009; London, UK. *Awarded the First Prize for poster presentation.*

<u>Vasilopoulou E</u>, Loubière LS, McCabe CJ, Franklyn JA, H Heuer, M Trajkovic, Kilby MD and Chan SY. The thyroid hormone transporter MCT8 affects human and mouse fetoplacental development. Oral presentation at the **80th American Thyroid Association Meeting**, September 2009; Palm Beach, FL, USA

<u>Vasilopoulou E</u>, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. Human primary cytotrophoblasts from normal and IUGR pregnancies respond differently to T3 treatment *in vitro*. Oral presentation at the **Trophoblast Day Meeting**, July 2009; Cambridge, UK

<u>Vasilopoulou E</u>, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. Human primary cytotrophoblasts from normal and IUGR pregnancies respond differently to T3 treatment *in vitro*. Oral presentation at the **British Maternal and Fetal Medicine Society Annual Meeting**, June 2009; Liverpool, UK

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. Human primary cytotrophoblasts from normal and IUGR pregnancies respond differently to T3 treatment *in vitro*. Poster presentation at the **56th Annual Meeting of the Society for Gynecologic Investigation**, March 2009; Glasgow, UK

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. Human primary cytotrophoblasts from normal and IUGR pregnancies respond differently to T3 treatment *in vitro*. Oral presentation at the **28th British Endocrine Society BES meeting**, March 2009; Harrogate, UK

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. The effects of the thyroid hormone transporter MCT8 on human placental development. Poster presentation at the **British Maternal and Fetal Medicine Society Annual Meeting,** June 2009; Liverpool, UK

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. The effects of the thyroid hormone transporter MCT8 on human placental development. Poster presentation at the **28th British Endocrine Society BES meeting**, March 2009, Harrogate, UK. *Awarded prize of best poster for the category "Thyroid".*

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. The effects of the thyroid hormone transporter MCT8 on human placental development. Poster presentation at the **79th Annual Meeting of the American Thyroid Association**, October 2008; Chicago, USA

Vasilopoulou E, Loubière LS, McCabe CJ, Franklyn JA, Kilby MD and Chan SY. The effects of the thyroid hormone transporter MCT8 on human placental development. Poster presentation at the **14th International Federation of Placenta Associations Meeting**, September 2008; Seggau castle, Austria. *Awarded YW Loke new investigator travel award.*

Chan SY, Loubière LS, Vasilopoulou E, McCabe CJ, Franklyn JA and Kilby MD. Expression of thyroid hormone transporters in human placenta and changes with intrauterine growth restriction (IUGR). Poster presentation at the **27th British Endocrine Society BES meeting,** April 2008; Harrogate, UK

Chan SY, Loubière LS, Vasilopoulou E, McCabe CJ, Franklyn JA and Kilby MD. Expression of thyroid hormone transporters in human placenta and changes with intrauterine growth restriction (IUGR). Poster Presentation at the **78th Annual Meeting of the American Thyroid Association,** October 2007; New York, USA

Chan SY, Loubière LS, Vasilopoulou E, McCabe CJ, Franklyn JA and Kilby MD. Expression of thyroid hormone transporters in human placenta and changes with intrauterine growth restriction (IUGR). Poster presentation at the **13th International Federation of Placenta Associations Meeting,** August 2007; Toronto, Canada